(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 896 002 A1

(12) EUROPEAN PATENT APPLICATION
published in accordance with Art. 158(3) EPC

(43) Date of publication:
10.02.1999 Bulletin 1999/06

(21) Application number: 98901043.4

(22) Date of filing: 29.01.1998

(51) Int. Cl.[6]: C07K 14/705, C12N 15/12, G01N 33/50

(86) International application number:
PCT/JP98/00370

(87) International publication number:
WO 98/32771 (30.07.1998 Gazette 1998/30)

(84) Designated Contracting States:
DE FR GB IT

(30) Priority: 29.01.1997 JP 15118/97
29.08.1997 JP 234544/97

(71) Applicant:
TORAY INDUSTRIES, INC.
Tokyo 103 (JP)

(72) Inventors:
- KAINOH, Mie
Kanagawa 251 (JP)
- TANAKA, Toshiaki
kanagawa 249 (JP)

(74) Representative: Kador & Partner
Corneliusstrasse 15
80469 München (DE)

(54) CHIMERIC PROTEINS, HETERODIMER COMPLEXES THEREOF AND SUBSTITUTE FOR PLATELET

(57) Integrin-immunoglobulin chimeric protein heterodimer complexes in which the α-chain and β-chain of integrins have been associated in a stable state. These complexes are not only usable as drugs as such but also applicable to the assaying of binding of integrins to ligands and the detection of substances binding to integrins or those inhibiting binding of integrins to ligands. These comnplexes are also usable as diagnostic agents. It has been found that integrins isolated in a stably associated structure would bind to extracellular matrixes under physiological conditions in the presence of plasma components, which indicates that integrins and, in its turn, extracellular matrix receptors might be usable as substitutes for platelets.

Printed by Xerox (UK) Business Services
2.16.7/3.6

Description

Technical Field:

[0001] The present invention relates to chimeric proteins consisting of an integrin and an immunoglobulin, their heterodimer complexes, a production process thereof, their applications as drugs and reagents, etc. Furthermore, the present invention relates to medicinal application of isolated extracellular maxtrix receptors such as integrin-immunoglobulin chimeric protein heterodimer complexes, as platelet substitutes.

Background Arts:

[0002] Various cells have receptors which mediate the adhesion between a cell and a cell and receptors which mediate the adhesion between a cell and an extracellular matrix, and these receptors play important roles in immune reaction, inflammatory reaction, development, morphogenesis, wound healing, hemostasis, cancerous metastasis, etc. By separating and identifying the receptors which participate in these phenomena, the existence of so-called cell adhesion molecules has been clarified. Many of the molecules identified one after another are classified in reference to their structural features into integrin superfamily, immunoglobulin superfamily, select in family, cadherin family, etc. (Corlos, T. M. and Harlan, J. M., Blood, 84, 2068-2101 (1994)). Of these families, the immunoglobulin superfamily, selectin family and cadherin family mediate mainly the adhesion between a cell and a cell, while the integrin superfamily is the so-called extracellular matrix receptors which mediate the adhesion to extracellular matrices such as fibronectin and collagens. In addition, extracellular matrix receptors which do not belong to any of these adhesion molecule families include CD26 (DDPIV), CD44, GPIV, GPVI, GPIb-vWF, etc. CD26 is a receptor for collagens, and CD44 is a receptor for hyaluronic acid, fibronectin and collagens ("Adhesion Molecules" p. 32-42, Masayuki Miyasaka (1991), Medical View (in Japanese)). Furthermore, it is reported that among the membrane glycoproteins (GPs) existing on platelets, GPIV, GPVI, GPIb-vWF, etc. are also collagen receptors ("Platelet Receptors", p. 119-132, Minoru Ohkuma et al., (1992), Kinpodo (in Japanese)).

[0003] A receptor belonging to the integrin superfamily has a heterodimer complex structure in which two subunits, α-chain and β-chain as mutually different membrane proteins are associated with each other non-covalently (Hynes, R. O., Cell, 48, 549-554 (1987)). In the past, the integrin superfamily was classified into three subfamilies; β1 integrin, β2 integrin and β3 integrin. Later, new β chains and α chains were discovered one after another, and presently eight β chains (β1, β2 β3, β4, β5, β6, β7 and β8 and fifteen α chains (α1, α2, α3, α4, α5, α6, α7, α8, α9, αv, αL, αM, αX, αIIb and αE) have been identified (Elner, S. G. and Elner, V. M. Inv. Ophtal. Vis. Sci., 37, 696-701 (1996)). It is known that each β chain is associated with one to eight α chains, and as a result, 21 pairs of an α chain and a β chain, i.e., integrin molecules have been identified (Elner, S. G. and Elner, V. M., Inv. Ophtal, Vis. Sci., 37, 696-701 (1996)). They include α 4β1 (VLA-4, β1 integrin), αLβ2 (LFA-1, β2 integrin), αMβ2 (Mac-1, β2 integrin), αIIbβ3 (GPIIb/IIIa, β3 integrin), etc. now targeted for drug development (Drug and Market Development, 6, 201-205 (1995)). Many other integrins are also expected to have relations with diseases.

[0004] The heterodimer complex structure of an integrin plays an important role in binding to a ligand (Hynes, R. O., Cell, 48, 549-554 (1987)). For example, it is estimated that the ligand binding region on an integrin consists of both an α chain and a β chain (Hynes, R. O., Cell, 69, 11-25 (1992)). The fact that integrins having the same α chain but associated with a different β chain, or integrins having the same β chain but associated with a different α chain are respectively different in substrate specificity (Elner, S. G. and Elner, V. M., Inv. Ophtal, Vis. Sci. 37, 696-701 (1996)) supports this assumption. On the other hand, it was reported that the α chains of some integrins have an sequence called an I domain consisting of about 180 amino acids inserted in the molecule, and data suggest that the I domain only could be bound to a ligand were reported (Ueda, T. et al., Proc. Natl. Acad. Sci. USA, 91, 10680-10684 (1994)). However, it was also reported that the I domain of an α domain and the integrin as its original heterodimer complex are different in the style of binding to a ligand (Kamata, T. and Takada, Y., J. Biol. Chem., 269, 26006-26010 (1994)). It is also not clarified yet whether such parameters as specificity and affinity to a ligand are identical. It is not reported that in the case of an integrin not containing the I domain, for example, in the case of α4β1 a partial structure only is bound to a ligand.

[0005] If any integrin isolated and prepared retains its heterodimer complex structure, hence the ligand binding capability, it can be used for studying the style of binding to a ligand in a state close to nature. Furthermore, it can be used as it is as a drug and can also be used as a reagent for measuring the amount of a ligand in tissue or serum or as a material for searching for adhesion inhibiting compounds very usefully. However, isolating and preparing an integrin with its function retained is said to be very difficult. One reason is that since the association between an α chain and a β chain of an integrin is maintained non-covalently as desribed before, they are easily dissociated during isolation and preparation. Since an integrin is a membrane protein, the necessity of using a surfactant, etc. for solubilization is considered to be a large cause in the dissociation of the complex. In other words, the non-covalent preservation of functional structure inhibits the preparation of such an integrin.

[0006] Inspite of the difficulty as described above, some cases were reported, in which an integrin heterodimer complex was isolated and prepared with its function retained. For cases of α2β1, α5β1 and αvβ3, it was reported that the binding to a ligand can be determined by letting a liposome incorporate an integrin purified by using affinity column chromatography (Santoro, S. A. et al., Biochem. Biophys. Res. Comm., 153, 217-223 (1988), Pytela, R. et al. Cell, 40, 191-198 (1985), Pytela, R. et al., Method Enzymol., 144, 475-489 (1987)). For other cases, it was that if purified α5β1 or αvβ3 is coated on a plate, a peptide which inhibits the cell adhesion through the integrin can be selected (Koivunen, E. et al., J. Biol. Chem., 268, 20205-20210 (1993), Healy, J. M. et al., Biochemistry, 34, 3948-3955 (1995)). For further other cases, it was reported that if purified αvβ3 or α4β1 is coated on a plate, the binding to a ligand can be determined (Charo, I. F. et al., J. Cell Biol., III, 2795-2800 (1990), Makarem, R. et al., J. Biol. Chem., 269, 4005-4011 (1994), Paul Mould, A. et al., J. Biol. Chem., 269, 27224-27230 (1994)). For a still further other case, it was reported that if an extracellar portion of αIIbβ3 heterodimer complex prepared by gene manipulation is coated on a plate through a complex specific antibody, the binding to a ligand can be determined (Gulino, D. et al., Eur. J. Biochem., 227, 108-115 (1995)). These cases suggest that to exert the function of a purified integrin, its heterodimer complex must be bound to or included in any carrier. The reason why a carrier is considered to be necessary is that since a heterodimeter complex is associated non-covalently in a solution, it tends to be dissociated and as a result, cannot retain its fuctional structure. In the finally stated case, only a molecule with a heterodimer complex structure is selected using a complex specific anti body, in a design to determine the binding in a state where both the chains are not dissociated from each other.

[0007] As a case requiring no carrier, it was reported that purified α1β1, or α2β1 allows the determination of the binding to a ligand dependent on high concentration of metal ions even without using any carrier (Pfaff, M. et al., Eur. J. Biochem., 225, 975-984 (1994)). In this case, the surfactant added in the process of purification plays a role similar to that of a liposome, acting as a carrier. For a further other case, it was reported that an extracellar of αMβ2 heterodimer complex prepared by using gene manipulation is bound to a ligand (Berman, P. W. et al., J. Cell Biochem., 52, 183-195 (1993)). These cases do not suggest the necessity of any carrier as described before, but the disadvantage that the association of molecules in a heterodimer complex is retained non-covalently is not improved.

[0008] As a still further other case, a chimeric protein consisting of αd and an immunoglobulin is disclosed (Japanese Patent Laid-Open (Kokai) No. 8-507933), but only the result of immune precipitation is reported, without examining the binding to a ligand. Furthermore, since a β chain is not expressed in the chimeric protein as an immunoglobulin, the binding between an α chain and a β chain remains non-covalent.

[0009] The above facts suggest that any integrin with an α chain and a β chain structurally stably associated and with its function retained has never been successfully prepared. That a complex structure is unstable restricts the use of its molecule.

[0010] Of the molecules belonging to the integrin superfamily, integrin α2β1 is an extracellular matrix receptor found to be expressed in T cells, platelets, etc. activated for long time. However, it was reported that the α2β1 on the cell surfaces of platelets and fibroblasts is bound to collagens only and that the α2β1 on the surfaces of vascular endothelial cells is bound to both collagens and laminins (Elices, M. J. et al., Proc. Natl. Acad. Sci. USA, 86, 9906-9910 (1989)), and it is speculated that the function of α2β1 becomes different, depending on cells.

[0011] In relation to the conditions of diseases, there are reports to suggest that integrin α2β1 plays an important roll for wound healing and cancerous metastasis (Shiro, J. A. et al., Cell, 67, 403-410 (1991), Chen, F. et al., J. Exp. Med., 173, 1111-1119 (1991), Chan, B. M. C. et al., Science, 251, 1600-1602 (1991)). Furthermore, it was reported that from the analysis of platelet function of patients with bleeding tendency, the adherence of platelets and collagens through integrin α2β1 has close relation with the first step of hemostasis and thrombosis process (Nieuwenhuis, H. K. et al., Nature, 318, 470-472 (1985)). Though the relations of integrin α2β1 with conditions of diseases are suggested like this, any medical application of using the integrin α2β1 protein and other isolated extracellular matrix receptor proteins under physiological ion condition or in the presence of plasma components has not been examined.

[0012] On the other hand, the necessity for artificial substitutes of platelets used as blood preparations in the clinical field is growing, and various attempts have been reported (Progress of Medicine 179, 406-407 (1996), Clinical Blood 37, 1353-1361 (1997) (respectively in Japanese)). However, they are not yet practically available.

Disclosure of the Invention:

[0013] The present invention relates to chimeric proteins in which the α chain and β chain of an integrin are combined with the heavy chain or light chain of an immunoglobulin, their heterodimer complexes, a production process thereof, a method for testing the binding of an integrin-immunoglobulin chimeric protein heterodimer complex to a ligand and a cell, substances bound to an integrin obtained by using the method, a method for searching for a substance inhibiting the binding between an integrin and a ligand using the integrin-immunogobulin chimeric protein heterodimer complex, substances for inhibiting the binding, and the application of integrin-immunoglobulin chimeric protein heterodimer complexes as drugs and reagents. Furthermore, the present invention relates to platelet substitutes containing an integrin-immunoglobulin chimeric protein heterodimer complex or any other isolated extradlular matrix receptor as an active

ingredient.

Brief Description of the Drawings:

[0014]

Fig. 1 shows that α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex is bound to VCAM-1 expressing cell, and that the binding is inhibited by an anti-integrin antibody or EDTA, a cationic cheating agent.
Fig. 2 shows that α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex is bound to CS-1 peptide, and that the binding is Inhibited by an anti-integrin antibody or EDTA, a cationic cheating agent.
Fig. 3 shows that the binding between α4 • IgG heavy chain-β1 IgG heavy chain chimeric protein heterodimer complex and CS-1 piptide is inhibited by GPEILDVPST, and is not inhibited by any other peptide.
Fig. 4 shows that α2 • IgG heavy chain-β1gG heavy chain chimeric protein heterodimer complex is bound to a collagen, and that the binding is inhibited by an anti-integrin antibody and EDTA, a cationic cheating agent.
Fig. 5 shows that α2 • IgG heavy chain-β1 IgG heavy chain chimeric protein heterodimer complex liposome is bound to a collagen in the presence of plasma.
Fig. 6 shows that the binding of α2 • IgG heavy chain-β1IgG heavy chain chimeric protein heterodimer complex liposome to a collagen is inhibited by an anti-integrin antibody or EDTA, a cationic chelating agent.

The Best Embodiments of the Invention:

[0015] The extracellular matrix receptors In the present invention refer generally to the receptors which mediate the adhesion between a cell and an extracellular matrix. The receptors include the integrin superfamily having a heterodimer complex structure in which an α chain and a β chain are non-covalently associated with each other as two membrane proteins (Corlos, T. M. and Harlan, J. M. Blood, 84, 2068-2101 (1994)), and other receptors such as CD26 (DDPIV), CD44, GPIV, GPVI, GPb-vWF, etc. The integrins in the present invention refer to molecules belonging to the integrin superfamily, and also include the isomers of the molecules belonging to the family. The α chains of the present invention include 15 α chains, i.e., α1, α2, α3, α4, α5, α6, α7, α8, α9, αv, αL, αM, αX, αllb and αE, and among them, α4 and α2 are preferable, though preferable α chains are not limited to them. The β chains of the present invention include eight β chains, i.e., β1, β2, β3, β4, β5, β6, β7and β8 and among them, β1 is preferable, though preferable β chains are not limited to it. The integrin molecules as pairs respectively consisting of an α chain and a β chain include the twenty one integrins stated in Elner, S. G. and Elner, V. M., Inv. Ophtal. Vis. Sci., 37, 696-701 (1996), though not limited to them.

[0016] A chimeric protein consisting of the α chain of an integrin and the heavy chain or light chain of an immunoglobulin refers to a molecule in which the extracellular region of the α chain of an integrin is bound to the constant region of the heavy chain or light chain contained an immunoglobulin. In this case, a chimeric protein in which N terminus side of the protein is integrin molecule and then connected to an immunoglobulin molecule side by side is preferable. A chimeric protein consisting of the β chain of an integrin and the heavy chain or light chain of an immunoglobulin refers to a molecule in which the extracellular region of the β chain of an integrin is bound to the constant region of the heavy chain or light chain contained in an immunoglobulin. Also in this case, a chimeric protein in which N terminus side of the protein is an integrin molecule and then connected to an immunoglobulin molecule side by side is preferable. In either case of α chain or β chain, a chimeric protein bound to the heavy chain of an immunoglobulin is preferable.

[0017] The isotype of the immunoglobulin to be bound to the α chain or β chain is not especially limited. Any of IgG, IgM, IgA and IgE can be used, but it is preferable to use IgG. The subclasses of IgG include IgG_1, IgG_2, IgG_3 and IgG_4, but it is preferable to use IgG_1. Furthermore, it is possible to use a molecule with a diner structure having a disulfide bond between molecules instead of the immunoglobulin.

[0018] In the present invention, a molecule in which a chimeric protein consisting of the α chain of an integrin and the heavy chain or light chain of an immunoglobulin and a chimeric protein consisting of the β chain of the integrin and the havy chain or light chain of the immunoglobuline are associated with each other is called an integrin-immunoglobulin chimeric protein heterodimer complex. In this case, a combination consisting of α chain • immunoglobulin heavy chain (which means a chimeric protein consisting of an α chain and the heavy chain of an immunoglobulin; hereinafter this applies) and β chain • immunoglobulin heavy chain, a combination consisting of α chain • immunoglobulin heavy chain and β chain • immunoglobulin light chain, and a combination consisting of α chain • imminoglobulin light chain and β chain • immunoglobulin heavy chain are preferable. A combination consisting of α chain • immunoglobulin heavy chain and β chain • immunoglobulin heavy chain is more preferable.

[0019] In the integrin-immunoglobulin chimeric protein heterodimer complex of the present invention, the α chain can be α1, α2, α3, α4, α5, α6, α7, α8, α9, αv, αL, αM, αX, αllb or αE, and the β chain can be β1, β2, β3, β4, β5, β6, β7 or

β8. It is preferable that the α chain is α4 or α2 and that the β chain is β1, though preferable chains are not limited to them.

[0020] The process for preparing an integrin-immunoglobulin chimeric protein heterodimer complex is described below, but the process is not limited thereto.

[0021] A DNA coding for the α chain and β chain of an integrin can be obtained using the information of known cDNA sequences by such a method as gene amplification based on the PCR method, cDNA cloning or DNA synthesis. For example, the DNA sequences of α4 and β1 are already reported in literature (Takada, Y. et al., EMBO J., 8, 1361-1368 (1989), Scott Argraves, W. et al., J. Cell Biol., 105, 1183-1190 (1987)). A DNA coding for the α chain and β chain of an integrin can also he obtained by the expression cloning using an antibody, etc. For binding to a DNA coding for the constant region of an immunoglobulin, it is desirable to take out a DNA coding for the extracellular portions only of the α chain and β chain of an integrin. For this purpose, it is preferable to use the PCR method and DNA synthesis. The extracellular portion of either an α chain or β chain refers to the polypeptide sequence on the N terminus side from the portion speculated to be the transmembrane portion. Its partial sequence can also be used as far as the ligand binding capability is retained, but it is preferable to use most of the portion considered to be an extracellular region. For taking out a DNA, it is necessary to adjust for adaptation of frames after linking to a DNA coding for an immunoglobulin. For example, this can be achieved by modifying the primer when a DNA fragment is taken out by the PCR method. In this case, it is desirable to design for ensuring that amino acid modification is not caused by the base substitution of the primer. However, amino acid substitution is allowed as far as the function of the chimeric protein is not changed. For obtaining a DNA by chemical synthesis, the purpose can be achieved by designing a sequence to ensure the linking to a DNA coding for an immunoglobulin. In the case of cDNA, a DNA capable of being bound to a DNA coding for an immunoglobulin can be prepared by using DNA fragmentation and a synthetic DNA.

[0022] Then, a DNA coding for an immunoglobulin is prepared. In the present invention, it is desirable to use DNAs coding for the heavy chain and light chain of a human immunoglobulin, but DNAs coding for an immunoglobulin of another animal species can also be used. The preparation of a DNA coding for human IgG is already reported (Ellison, J. W. et al., Nucleic Acids Res., 10, 4071-4079 (1982)), but the preparation is not limited to this method. Any method similar to the above mentioned method for preparing DNAs coding for the α chain and β chain of an integrin can also be used. In the present invention, for the heavy chain of a human immunoglobulin, it is preferable to use a genomio DNA, but a cDNA can also be used. As the DNA for the heavy chain of a human immunoglobulin, it is preferable to use a portion coding for the hinge region, CH2 region or CH3 region, but a DNA coding for the entire constant region of CH1 - CH3 can also be used. For the light chain of an immunoglobulin, a DNA coding for the CL region is used. Finally, a DNA coding for the extracellular portion of an α chain or β chain and a DNA coding for the constant region of human immunoglobulin heavy chain are linked with in frame. The obtained DNA codes for a polypeptide starting from the methionine of translation initiation and having the signal sequence of the α chain or β chain of an integrin, its extracellular region and the constant region of human immunoglobulin heavy chain linked in this order.

[0023] The DNA coding for a chimeric protein consisting of the α chain of an integrin and the heavy chain or light chain of an immunoglobulin, or the DNA coding for a chimeric protein consisting of the β chain of an integrin and the heavy chain or light chain of an immunoglobulin respectively obtained in the above is functionally linked in a proper expression control sequence, to obtain a recombinant vector. The general methods concerning gene recombination such as the method for preparing the recombinant vector, the method for transfecting it into a cell are described in a published book ("Molecular Cloning", Sambrook et al., (1989) Cold Spring Harbor Lab. Press, New York), but the methods are not limited to those stated there. In the present invention, it is desirable to use an expression control sequence suitable for protein expression in animal cells. For example, for manifestation of insect cells, polyhedrin promotor, p10 promotor, etc. are generally used as expression control sequences, and for expression of other animals' cells, SRα promotor, cytomegalovirus derived promotor, simian virus 40 derived promotor, polyhedrin promotor, p10 promotor, etc. are used. However, the expression control sequences are not limited to them. In the present invention, it is preferable to use SRα promotor.

[0024] If the obtained recombinant vector is transfected into a cell, a cell capable of producing an integrin-immunoglobulin chimeric protein heterodimer complex can be obtained. In this case it is preferable to use an animal derived cell as a host. For example, COS cell (simian renal cell), CHO cell (Chinese Hamster ovarian cell), Sf9 (insect cell), etc. are generally used as hosts. Furthermore, myeloma cells such as P3U1 and Y3 can also be used. Other established cell lines and cloned cells can also be used, but the cells used as hosts are not limited to them. In the present invention, it is preferable to use a CHO cell.

[0025] It is known that the methods for transfecting a recombinant vector into a cell include the lipofectin method, calcium phosphate method, electroporation method, etc., and any of the methods can be used. The method is not limited to them. It is preferable that when a cell is transfected by using a recombinant vector, a recombinant vector for expression of a chimeric protein consisting of the α chain of an integrin and the heavy chain or light chain of an immunoglobulin and a recombinant vector for expression of a chimeric protein consisting of the β chain of the integrin and the heavy chain or light chain of the immunoglobulin are transfected into the cell one after another using different drug resistance

markers. The recombinant vectors can be transfected in any order or simultaneously. It is desirable that the two recombinant vectors to be transfected are vectors for expression of a combination consisting of α chain • immunoglobulin heavy chain (which means a chimeric protein consisting of an α chain and the heavy chain of an immunoglobulin; hereinafter this applies) and β chain • immunoglobulin heavy chain, or α chain • immunoglobulin heavy chain and β chain • immunoglobulin light chain, or α chain • imminoglobulin light chain and β chain • immunoglobulin heavy chain. Any of these combinations can be adopted, but a combination of recombinant vectors for expression of α chain • immunoglobulin heavy chain and β chain • immunoglobulin heavy chain is desirable.

[0026] In any transfection method and any combination of vectors, it is important to select a cell which is transfected by the two recombinant vectors and produces a chimera protein consisting of an α chain and the heavy chain or light chain of an immunoglobulin and a chimera protein consisting of a β chain and the heavy chain or light chain of an immunoglobulin simultaneously almost by the same amounts. This can be achieved by measuring the amounts of the chimeric protein consisting of an α chain and the heavy chain or light chain of an immunoglobulin and a chimeric protein consisting of a β chain and the heavy chain or light chain of an immunoglobulin produced in the cultured supernatant solution of the cell transfected by the recombinant vectors. For measurement, for example, the transfected cell can be cultured in a medium containing ^{35}S according to any publicly known method, for labeling the proteins, and the amounts of the chimeric protein consisting of an α chain and the heavy chain or light chain of an immunoglobulin and a chimeric protein consisting of a β chain and the heavy chain or light chain of an immunoglobulin existing in the cultured supernatant solution can be estimated by immunoprecipitation using an anti-α chain antibody or an anti-β chain antibody respectively. As another method, the amounts the chimeric protein consisting of an α chain and the heavy chain or light chain of an immunoglobulin and a chimeric protein consisting of a β chain and the heavy chain or light chain of an immunoglobulin existing in the cultured supernatant solution can be estimated according to the ELISA method using an anti-human immunoglobulin antibody and an anti-α chain antibody or an anti-β chain antibody. Anyway, it is preferable to select a clone which produces almost the same large amounts of the chimeric proteins of the α and β chains in the culture supernatant , for preparing an integrin-immunoglobulin chimeric protein heterodimer complex. The methods for labeling proteins, the methods of immunoprecipitation and the general methods of ELISA are described in a published book ("Antibody" Harlow, E., and Lane, D. (1988), Cold Spring Harbor Lab. Press, New York), but the methods are not limited to them. Any other method can also be used for detecting chimeric proteins.

[0027] The obtained transfected cell can be cultured according to a general cell culture method, to produce an integrin-immunoglobulin chimeric protein heterodimer complex. It is preferable that the medium contains about 5% of serum of a low immunoglobulin concentration, but any generally known serum-containing medium or a serum-less medium can also be used. After completion of cell culture, the cells and solid matter are removed by such operation as centrifugation, and the culture supernatant containing an integrin-immunoglobulin chimeric protein heterodimer complex is collected.

[0028] It can be estimated that the cultured supernatant solution contains not only the integrin-immunoglobulin chimeric proteins in which the chimeric protein consisting of an α chain and the heavy chain or light chain of an immunoglobulin and a chimeric protein consisting of a β chain and the heavy chain or light chain of an immunoglobulin form a heterodimer complex, but also the chimeric protein consisting of an α chain and the heavy chain or light chain of an immunoglobulin and a chimeric protein consisting of a β chain and the heavy chain or light chain of an immunoglobulin which do not form the heterodimer complex. However, since the molecules other than the heterodimer complex cannot be bound to a ligand, the supernatant solution can be used as a reagent for testing the binding to a ligand or cell, or searching for a substance inhibiting the binding between an integrin and a ligand, or for searching for a substance capable of being bound to an integrin, or for measuring the ligand amount of an integrin. These methods of utilization are basically the same as those for using a purified integrin-immunoglobulin chimeric protein heterodimer complex described later.

[0029] An integrin-immunoglobulin chimeric protein heterodimer complex can be purified by an established method using a protein A column chromatography by use of the nature of the immunoglobulin portion. Furthermore, affinity chromatography using an antibody against the α or β chain can a so be used. Moreover, the purification can also be achieved by affinity chromatography with a ligand bound to a carrier. General chromatographic methods can also be used in combination for the purification. If publicly known cases in which integrin molecules are purified by these methods (Pytela, R. et al., Methods Enzymol., 144, 475-489 (1987), Santoro, S. A. et al., Biochem. Biophys. Res. Comm., 153, 217-223 (1988), Charo, I.F. et al., J. Cell Biol., 111, 2795-2800 (1990), Makarem, R. et al., J. Biol. Chem., 269, 4005-4011 (1994), Pfaff, M. et al., Eur. J. Immunol., 225, 975-984 (1994), Gulino, D. et al., Eur. J. Biochem., 227, 108-115 (1995), etc.) are applied, the purification of an integrin-immunoglobulin chimeric protein heterodimer complex can be achieved.

[0030] A purified integrin-immunoglobulin chimeric protein heterodimer complex can be identified as a protein showing at least one band under non-reducing condition and at least two bands under reducting condition by SDS-PAGE. It can also be confirmed from it, that the heterodimer is linked by the disulfide bond between immunoglobulin heavy chains. It sometimes occurs that a plurality of bands are detected under reduction, but this is considered to be probably

because intramolecular clearage of the α chain has occurred. Especially, with α4, this phenomenon is known (Hemler, M. E. et al., J. Biol., Chem., 262, 11478-11485 (1987)). Furthermore, it can be confirmed by the Western blotting method that the respective bands indicate chimeric proteins. As another method, it can be confirmed by said ELISA method combining an anti-α chain anti body, anti-β chain antibody and anti-human immunoglobulin antibody, that the obtained molecule is an integrin-immunoglobulin chimeric protein heterodimer complex. That is, the molecule can be identified as a protein molecule with epitopes for all the antibodies. As a further other method, an integrin-immunoglobulin chimeric protein heterodimer complex can also be identified by immunoprecipitation. In this case, if the purified protein is labeled by ^{35}S, or ^{125}I or biotin, etc. according to any known method, and immunoprecipitated using an anti-α chain antibody, anti-β chain antibody and anti-human immunoglobulin antibody, the same electrophoretic pattern can be obtained in every case. So, it can be confirmed that the integrin-immunoglobulin chimeric protein heterodimer complex has the intended structure. Furthermore, even if a condition to dissociate the integrin complex on a cell membrane such as the coexistence of EDTA or boiling in the presence of SDS is applied, the immunoprecipitation pattern is not changed. So, it can be confirmed that the obtained integrin-immunoglobulin chimeric protein heterodimer complex is a structurally stabilized complex. The methods for confirming an integrin-immunoglobulin chimeric protein heterodimer complex are not limited to those stated above.

[0031] The binding between a prepared integrin-immunoglobulin chimeric protein heterodimer complex and a ligand can be tested as described below. After a ligand and an integrin-immunoglobulin chimeric protein heterodimer complex are brought into contact with each other, to make a mixture, the amount of the integrin-immunoglobulin chimeric protein heterodimeter complex bound to the ligand or the amount of the ligand bound to the integrin-immunoglobulin chimeric protein heterodimeter complex is measured. The amount of an integrin-immunoglobulin chimeric protein heterodimer complex can be measured by labeling the complex itself by a fluorescent dye or enzyme or radio isotope, etc. The amount of a ligand can also be measured by any similar method. A detection method such as SPA (Amasham) can also be used for the measurement. Furthermore, any reagent which can recognize a complex or ligand labeled by a fluorescent dye, enzyme or radioisotope, etc. can also be used for the measurement. The reagent for recognizing an integrin-immunoglobulin chimeric protein heterodimer complex can, for example, be an anti-human immunoglobulin antibody. In this test, it is preferable to bind the molecule to be detected, to any carrier such as a bead or plate. As a ligand, its entire molecule can be used, but a portion retaining the binding activity to an integrin can also be taken out for use. For example, for integrin α4β1 or integrin α2β1, its ligand, fibronectin or collagen or its peptide fragment bound to a carrier can also be used.

[0032] Methods similar to the above can be used to test the binding between an integrin-immunoglobulin chimeric protein heterodimer complex and cells. The amount of the cells bound to a complex can be measured by labeling the cells by a fluorescent dye or radioisotope or using a reagent reacting with the cells, for example, an antibody reacting with a surface antigen. If something like a tissue section is used instead of cells, the amount of the bound integrin-immunoglobulin chimeric protein heterodimer complex is measured by any of the above mentioned methods.

[0033] The methods for examining the binding between an integrin-immunoglobulin chimeric protein heterodimer complex and a ligand or cell described above can be used for obtaining a substance inhibiting the binding between an integrin and a ligand, for example, for obtaining an antibody, polypeptide, peptide or low molecular weight compound. It is preferable to premix a sample and an integrin-immunoglobulin chimeric protein heterodimer complex, and then to measure the amount of the integrin-immunoglobulin chimeric protein heterodimer complex bound to a ligand in any of the above mentioned measuring systems. If the amount of the bound integrin-immunoglobulin chimeric protein heterodimer complex is lowered by adding a certain sample, it can be judged that the sample has inhibitory activity. However, in this sytem, a substance with metal ion cheating action or a substance with surfactant action, etc. may give a false positive result. The sources of samples used include the following integrin bound substances, peptide fragments of ligands, their derivatives, marketed compounds, etc., but are not limited to them.

[0034] A case where a purified integrin was coated on a plate to search for a peptide to be bound was reported (Healy, J. M. et al., Biochemistry 34, 3948-3955 (1995)). Even if the integrin-immunoglobulin chimeric protein heterodimer complex obtained in the present invention is used, a substance to be bound to an integrin can be similarly searched for. Especially when the chemiric protein heterodimer complex of the present invention is used, the operation to remove the non-specifically bound substances can be effected under more severe conditions. So, the operation can be simplified advantageously. Furthermore, since the complex is not dissociated during operation, a bound substance can be selected more specifically advantageously. Known sources suitable for selecting bound substances include a phage peptide library (e.g., Scott, J. K. and Smith, G. P., Science, 249, 386-390 (1990)) and a DNA oligomer library (e.g., O'Connel, D. et al., Proc. Natl. Acad. Sci. USA, 93, 5883-5887 (1996), but in the present invention, it is preferable to use the former.

[0035] Furthermore, the method of testing the binding between an integrin-immunoglobulin chimeric protein heterodimer complex and a ligand or cell can also be used as a method for measuring the amount of an integrin ligand in a body fluid or tissue.

[0036] Moreover, the integrin-immunoglobulin chimeric protein heterodimer complexes of the present invention can

also be used as drugs. The present invention has clarified that integrins and other isolated extracellular matrix receptors can be used as platelet substitutes.

[0037] An extracellular matrix receptor preferably used as a platelet substitute is an integrin. The α chain of the integrin can be $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\alpha 8$, $\alpha 9$, αv, αL, αM, αX, αIIb or αE, and among them, $\alpha 2$ is preferable. The β chain can be $\beta 1$, $\beta 2$, $\beta 3$, $\beta 4$, $\beta 5$, $\beta 6$, $\beta 7$or $\beta 8$, and among them, $\beta 1$ is preferable. Integrin $\alpha 2\beta 1$ is more preferable. The receptor source for isolation can be a tissue or cell expressing an extracellular matrix receptor, or a dissolved membrane fraction of a receptor expressing cell prepared by gene manipulation, etc. It is more preferable to design for obtaining a soluble protein by modifying a receptor gene by gene recombination, and to use the cultured supernatant solution of the cells capable of producing it, as a source. Furthermore in the design of the soluble protein, it is preferable that the functional structure of the extracellular matrix receptor is retained. For example, it is desirable to use an integrin-immunoglobulin chimeric protein heterodimer complex obtained by modifying the heterodimer structure of an integrin to allow its α and β chains to be covalently associated with each other. As the integrin-immunoglobulin chimeric protein heterodimer complex, it is preferable that the α chain of the integrin is $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\alpha 8$, $\alpha 9$, αv, αL, αM, αX, αIIb or αE, and among them, $\alpha 2$ is more preferable. Furthermore, it is preferable that the β chain is $\beta 1$, $\beta 2$, $\beta 3$, $\beta 4$, $\beta 5$, $\beta 6$, $\beta 7$ or $\beta 8$, and among them, $\beta 1$ is more preferable. It is further more preferable that the α chain is $\alpha 2$ and that the β chain is $\beta 1$. The platelet substitute of the present invention is described below mainly in reference to a typical extracellular matrix receptor, integrin $\alpha 2\beta 1$-immunoglolublin chimeric protein heterodimer complex, but the present invention is not limited thereto or thereby.

[0038] To confirm the applicability of a purified integrin-immunoglobulin chimeric protein heterodimer complex as a drug, the purified protein itself is used for examining its pharmacological activity. For obtaining higher capability of being bound to an extracellular matrix, it is more preferable to use an integrin-immunoglobulin chimeric protein heterodimer complex bound to a carrier such as a lipid or protein polymer, etc., but the present invent ion is not limited to this method.

[0039] For use as a platelet substitute, it is preferable to bind an integrin $\alpha 2\beta 1$-immunoglolublin chimeric protein heterodimer complex to a liposome covalently according to the method stated in a report (Martin, F. J. et al., Biochemistry, 20, 4229 (1981)). The carrier can also be any other drug carrier than a liposome as far as its use for drugs is permitted. If a liposome is used as the carrier, the liposome is prepared according to the composition and method stated in a published book "Preparation and Experiments of Liposomes (in Japanese)", Oku, N. (1994), Hirokawa Shoten), but a preferable method is such that the epitope bound to the extracellular matrix of an integrin $\alpha 2\beta 1$-immunoglolublin chimeric protein heterodimer complex is exposed outside the liposome membrane.

[0040] For confirming that an integrin $\alpha 2\beta 1$-immunoglolublin chimeric protein heterodimer complex is bound on the prepared liposome carrier, a flow cytometer is used. The reagents which can be used for recognizing the integrin $\alpha 2\beta 1$-immunoglolublin chimeric protein heterodimer complex include an anti-integrin $\alpha 2$ antibody, anti-integrin $\beta 1$ antibody, anti-human immunoglobulin antibody, etc. If the antibody used is fluorescently labeled, it can be used for determination directly, but if it is not fluorescently labeled, a secondary antibody which recognizes the immunoglobulin class of the animal species used for preparing the antibody is used as a fluorescent label. As a further other confirmation method, the integrin $\alpha 2\beta 1$-immunoglolublin chimeric protein heterodimer composite itself can be labeled by an enzyme or radioisotope, etc., for confirmation in proper combination with a color dye or radioactivity measuring instrument, etc.

[0041] To examine the extracellular matrix binding capability using an integrin $\alpha 2\beta 1$-immunoglolublin chimeric protein heterodimer complex liposome, it is preferable to suspend the integrin $\alpha 2\beta 1$-immunoglolublin chimeric protein heterodimer complex liposome into a buffer with a physiological cation concentration or plasma. The buffer with a physiological cation concentration refers to a buffer containing at least cations such as Mg ions or Ca ions and adjusted to about neutrality. The plasma is prepared by processing the blood collected in the presence of an anticoagulant, according to a general plasma preparation method. As the anticoagulant, for example, heparin or EDTA solution can be added by sufficient units. A marketed normal plasma, coagulation factor deficient plasma or serum, etc. can also be used. However, if the anticoagulant used lowers the cation concentration, cations are added to achieve a physiological concentration later. Then, the integrin $\alpha 2\beta 1$-immunoglolublin chimeric protein heterodimer complex liposome is mixed with an extracellular matrix or its fragment coated on a carrier for a certain time, to judge whether binding takes place. It is preferable that the coating of the extracellular matrix or its fragment as a solid phase is achieved by using a plastic plate, etc., but marketed beads for coating an extracellular matrix as a solid phase, etc. can also be used. When a collagen is used as the extracellular matrix, any animal species and type can be used. The binding reaction between an integrin $\alpha 2\beta 1$-immunoglolublin chimeric protein heterodimer complex liposome and an extracellular matrix is effected according to a general method adopted for observing the adherence reaction of platelets. In many cases, they are allowed to stand mainly in a static system for a certain time, to induce binding to the matrix, but it is preferable to apply a shaking or shear stress, etc.

[0042] The integrin $\alpha 2\beta 1$-immunoglolublin chimeric protein heterodimer complex liposome is bound to an extracellular matrix under the conditions as described above, and the amount of binding is measured by applying the above mentioned ELISA method using an anti-human immunoglobulin antibody. For more accurate determination, it is desirable to

immobilize the liposome bound to the matrix by 1% glutaraldehyde, etc. As another method than the ELISA method, for example, if a radio-labeled lipid is incorporated into the liposome beforehand, the amount of the liposome bound to the extracellular matrix can be obtained as radioactivity. Furthermore, to qualitatively judge the binding and covering degree to the extracellular matrix, a labeled antibody for recognizing the integrin α2β1-immunogloIublin chimeric protein heterodimer complex on the bound liposome can be combined with a color dye, etc., to dye the portions where the liposome is bound. It is more preferable that the generally used tissue antibody dyeing method is used to use a peroxidase labeled antibody against the integrin α2β1-immunogloIublin chimeric protein heterodimer complex and diaminobenzidine in combination, but the measuring method is not limited to it. As a further other method, the area covering the extracellular matrix can be obtained as a covering rate using an image processing analyzer.

[0043] Methods for examining the hemostasis of platelets include testing the adhering capability of platelets to the extracellular matrix and the agglutination capability induced by a collagen ("Handbook on the Examination of Blood Coagulation (in Japanese)", p. 65-78, Fukutake, M. and Fujimaki, M. (1987), Uchudo Yagi Shoten, Santro, S.A., Cell, 46, 913-920 (1986), Lethagen, S. and Rugarrn, P., Thrombo Haemost., 67, 185-186 (1982)). Especially the adhering capability of platelets to the extracellular matrix is an indicator of primary hemostasis. The adhering capability is evaluated by using blood as it is, or platelet rich plasma or platelets washed by a buffer with physiological ions. Therefore, whether or not the integrin α2β1-immunogloIublin chimeric protein heterodimer complex liposome obtained in the present invention can be a functional substitute of platelets can be judged in reference its binding capability and the level of the binding capability to the extracellular matrix in the existence of plasma components or at a physiological ion concentration.

[0044] If the binding capability of the integrin α2β1-immunogloIublin chimeric protein heterodimer complex liposome obtained in the present invention to the extracellular matrix in the presence of the plasma components is strong, it suggests that the liposome can be a platelet substitute. Therefore, it can be used as a therapeutic or preventive agent against the congenital and acquired bleeding tendency due to platelet abnormality, and also widely as a platelet transfusion substitute.

[0045] Similarly the integrin α2β1-immunogloIublin chimeric protein heterodimer complex liposome obtained in the present invention can be a therapeutic or preventive agent for conditions of diseases where vascular endothelial cell disorder is a problem. For example, it was reported that in the prognosis of PTCA (percutaneous coronary restenosis), the excessive accumulation of platelets on the extracellular matrix exposed by balloon catherter treatment triggers restenosis (Liu, M.W. et al., Circulation, 79, 1374-1378 (1989)). In Example 22, the effect of the integrin α2β1-immunogloIublin chimeric protein heterodimer complex liposome to cover the extracellular matrix was confirmed, and this effect can reduce the excessive accumulation of platelets to allow use also as a restenosis preventive. Furthermore, if the integrin α2β1-immunogloIublin chimeric protein heterodimer complex liposome is labeled by a medically allowable method, it can be used for monitoring the region of the extracellular matrix exposed by vascular endothelial cell injury, and furthermore, if a drug is enclosed in the liposome, it can also be applied to the targeting therapy for a local injured region.

[0046] When any integrin α2β1-immunogloIublin chimeric protein heterodimer complex liposome stated in the present invention is used as a platelet substitute, the administration paths include infusion, intravenous administration, etc., and it is usually used by being suspended in any physiologically suitable solution such as a salt solution or plasma, etc. It can be used alone or also in combination with another chimeric protein heterodimer complex with an extracellular matrix receptor or its immunoglobulin. It can also be used together with another drug containing total platelets. The dose is properly selected to suit the symptom, age, body weight, etc. and can be 0.1 mg to 10 g per day as the amount of the protein for an adult, being able to be administered at a time or in several times. It can also be mixed with a pharmaceutically allowed carrier or excipient, etc., to be applied locally to the injured region as an externally applied drug such as an ointment, liniment or plaster. In this case, the externally applied drug is prepared to be 1 ng/cm^2 to 1 mg/cm^2 as the amount of the protein per one time of coating.

Examples

[0047] To describe the present invention in more detail, examples are given below. The general methods of recombinant DNA experiments conformed to those stated in a published book ("Antibody", Harlow, E. and Lane, D. (1988), Cold Spring Harbor Lab. Press, New York).

Example 1

Construction of human IgG$_1$ heavy chain expression vector

[0048] As human IgG$_1$ genome gene, a clone identical with reported base sequence information (Ellison, J. W. et al., Nucleic Acids Res., 10, 4071-4079 (1982)) was acquired from a human genomic library (CLONTECH) using a hybridization cDNA probe based on the sequence information. This was used as the template DNA for PCR. As primers for

amplifying the DNA fragment containing the hinge region (H) and the constant region portions (CH2 and CH3) of human IgG_1 gene, a DNA oligomer shown in sequence No. 4 of the sequence table (hereinafter a sequence No. of the sequence table is simply called a sequence No.) with BamH I restriction site and a DNA oligomer shown in sequence No. 5 with Xba I restriction site were synthesized.

5'-GCGGATCCCGAGCTGCTGGAAGCAGGCTCAG-3' (Sequence No. 4)

5'- CCTCTAGACGGCCGTCGCACTCATTTA-3' (Sequence No. 5)

[0049] The template DNA, primers, dNTPs (an equimolar mixture of dATP, dCTP, dGTP and dTTT) and Taq polymerase (Takara) were mixed in a PCR buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin, pH 8.3), and in a thermal cycler (Perkin Elmer Cetus), the mixture was treated at 94 °C for 1 minute for DNA denaturation, at 58°C for 2 minutes for annealing the primers and at 72°C for 3 minutes for elongating the primers. This treatment was performed 30 cycles. The amplified DNA was digested by restriction enzymes BamH I and Xba I, and the DNA fragment was purified by 1% agarose gel according to a general method ("Antibody", Harlow, E. and Lane, D. (1988), Cold Spring Harbor Lab. Press, New York). It was linked, using a T4DNA ligase, with a large DNA fragment of pBluescriptSK(+) (STRATAGENE) purified and digested by restriction enzymes BamH I and Xba I. The plasmid DNA was used to transform Escherichia coli (JM109), and the transformant was selected, to obtain a plasmid DNA (IgG_1 Bluescript). Then, expression vector pcDL-SR α296 was digested by restriction enzyme BamH I, and blunted at the termini by T4DNA polymerase treatment, and a Not I linker was linked. The large DNA fragment obtained by digesting it by restriction enzymes Not I and Xho I and the small DNA fragment obtained by digesting IgG_1Bluescript by restriction enzymes Not I and Xho I were purified according to a general method, and linked by T4DNA ligase. It was transformed into Escherichia coli (HB101), and the transformant was selected, to obtain a plasmid DNA. Hereinafter this plasmid ($IgG_1SR\alpha$) is called human IgG_1 expression vector. In the following examples, since the basic protocol of gene manipulation is the same as above, the description will be simplified.

Example 2

Construction of integrin α4 • IgG heavy chain chimeric protein expression vector

[0050] The DNA fragment coding for the extracellular portion of integrin α4 was obtained by cloning based on reported cDNA sequence information (Takada, Y. et al., EMBO J., 8, 1361-1368 (1989)). The restriction site EcoR I of 1801-base-position of sequence No. 1, the restriction site Stu I of 112-base-position and the restriction site BamH I of 2949-base-position were used for linking the region from the N terminus translation initiation site to Stu I cut site as α4-1, the region from Stu I cut site to EcoR I cut site as α4-2, and the region from EcoR I detached site to BamH I detached site as α4-3. The detailed methods are described below.

[0051] The portion coding for α4-1 was designed to be cloned by linking the DNA oligomers of sequence Nos. 6 to 9, and the DNA oligomers shown in sequence Nos. 6 to 9 were synthesized. For the sequence Nos. 6 and 7, restriction site Xba I was added on the side to code for the N terminus, for linking to a vector. Furthermore, compared with the known sequence information, the bases at the 60-, 63- and 64-positions were substituted from C to T, C to A and C to G respectively, and the bases at the 112- and 114-positions were substituted frseom C to A and C to G respectively. Because of substitution at the 112- and 114-position, restriction site Stu I was inserted on the side to code for the N terminus of sequence Nos. 8 and 9. The 5' termini of the synthesized oligomers were phosphated and annealed, and were linked using T4DNA ligase. After completion of linking, restriction enzymes Xba I and Stu I were used for cutting, and electrophoresis was effected by 5% agarose (NuSieve GTGagarose, FMC) gel. The intended DNA fragment (α4-1) of about 120 bp was cut out and purified.

5'-CTAGACCACCATGTTCCCCACCGAGAGCGCATGGCTTGGCAAGCGACGCGCGAACCCGGCCCCGGA
GCTGCA-3' (Sequence No. 6)

5'-GCTTCGGGGCCCGGGTTCGCGCCTCGCTTGCCAAGCCATGCGCTCTCGGTGGGGAACATGGTGGT-3' (Sequence No. 7)

5'-CTCCGGCAGACGGTGATGCTGTTGCTGTGCCTGGGGGTCCCGACCGGCAGG-3'

(Sequence No. 8)

5'-CCTGCCGGTCGGGACCCCCAGGCACAGCAACAGCATCACCGTCTCCCGGAGTCGA-3'

(Sequence No. 9)

[0052] Then, the RNA of human osteosarcoma cell line MG63 (ATCC CRL 1427) as an integrin α4 expressing cell was separated, and PolyA(+)RNA was purified using oligo dT cellulose column (NEB). Based on it, a single stranded cDNA was synthesized using a reverse transcriptase (GIBCO), and used as the template for PCR. As primers for amplifying α4-2 and α4-3 DNAs, four DNA oligomers of sequence Nos. 10 to 13 with Pst I and Stu I restriction sites inserted (sequence No. 10) or BamH I restriction site inserted (sequence No. 13) were synthesized.

5'-CACTGCAGGCAGGCCTTACAACGTGGACACTCACACC-3' (Sequence No. 10)

5'-GCAGAAACCTGTAAATCAGCAG-3' (Sequence No. 11)

5'-GCATTTATGCGGAAAGATGTGC-3' (Sequence No. 12)

5'-CGGGATCCGTGAAATAACGTTTGGGTCTT-3' (Sequence No. 13)

[0053] The template cDNA, primers, dNTPs and Taq polymerase were mixed in a PCR buffer, and the mixture was treated by a thermal cycler at 94°C for 1 minute for DNA denaturation, at 58°C for 2 minutes for annealing the primers and at 72°C for 3 minutes for elongating the primers. This treatment was performed 30 cycles. The amplified DNA fragments of α4-2 and α4-3 were digested by Pst I and EcoR I respectively, or EcoR I and BamH I and sub-cloned into pBluescriptKS(+) (STRATAGENE), to prepare plasmid DNAs (hereinafter called α4-2 Bluescript and α4-3 Bluescript). Then, upstream of the α4-2 Bluescript, α4-1 was linked using Xba I and Stu I restriction sites, to prepare a plasmid DNA (hereinafter called α4-1-2 Bluescript).

[0054] The α4-1-2 Bluescript was digested by restriction enzyme Not I, and blunted at the termini by T4DNA polymerase treatment, being digested by restriction enzyme EcoR I, to prepare a small DNA fragment. The α4-3 Bluescript was digested by restriction enzymes EcoR I and BamH I, to prepare a small DNA fragment. The two small DNA fragments were simultaneously linked to a large DNA fragment obtained by digesting $IgG_1SR\alpha$ by restriction enzymes EcoR V and BamH I, to obtain a plasmid DNA. The obtained base sequence coding for integrin α4 · IgG heavy chain chimeric protein is shown as sequence No. 1. The plasmid (integrin α4 · IgGSRα) is hereinafter called integrin α4 · IgG heavy chain chimeric protein expression vector.

Example 3

Construction of β1 • IgG heavy chain chimeric protein expression vector

[0055] The RNA of human fibroblast cell line MRC5 (ATCC CCL 171) as an integrin β1 expressing cell was separated, and oligo dT cellulose column was used to purify PolyA (+)RNA. Based on it, a single stranded cDNA was synthesized using a reverse transcriptase, and used as the template for PCR. As primers, two DNA oligomers of sequence Nos. 14 and 15 with BamH I site (sequence No. 15) inserted on the side coding for C terminus were synthesized according to the sequence information (Cott Argraves, W. et al., J. Cell Biol., 105, 1183-1190 (1987)).

5'-GCGCAAAAGATGAATTTACAAC-3' (Sequence No. 14)

5'-GTGCGATCCTCTGCACCAGTGCGCACAC-3' (Sequence No. 15)

[0056] The template cDNA, primers, dNTPs and Taq polymerase were mixed in a PCR buffer, and treated by a thermal cycler at 94°C for 1 minute for DNA denaturation, at 57°C for 2 minutes for annealing the primers and at 72°C for 3 minutes for elongating the primers. This treatment was performed 30 cycles. The amplified DNA was blunted at the termini by T4DNA polymerase treatment, and digested by restriction enzyme BamH I. Then, the DNA fragment was purified. Subsequently, the DNA fragment obtained in PCR before was sub-cloned at the Sma I and BamH I sites of pBluescriptKS(+). A small DNA fragment purified by digesting it by restriction enzymes EcoR I and BamH I was inserted into a large DNA fragment of $IgG_1SR\alpha$ treated by restriction enzymes EcoR I and BamH I, to obtain a plasmid DNA. The obtained base sequence coding for β1 • IgG heavy chain chimeric protein is shown in sequence No. 2. The plasmid (integrin β1 • IgGSRα) is hereinafter called integrin β1 • IgG heavy chain chimeric protein expression vector.

Example 4

Transfection of α4 • IgG heavy chain chimeric protein expression vector and β1 • IgG heavy chain chimeric protein expression vector into animal cells, and their expression

[0057] Integrin β1 • IgGSRα as β1 • IgG heavy chain chimeric protein expression vector and pSV2dhfr (BRL) were mixed at a ratio of 10 : 1, and the mixture and lipofectin reagent (GIBCO BRL) were gently mixed and allowed to stand at room temperature for 15 minutes. The mixture was added dropwise to dihydrofolic acid reductase deficient CHO cells (ATCC CRL 9096). After 18 hours of dropwise addition, the mixture was cultured in a medium (10%FBS (GIBCO), nucleic acid-containing αMEM medium (GIBCO BRL)) for about 2 days, and the cells were dispersed by trypsin-EDTA treatment. The cells were suspended in a first selective medium (10% FBS-containing nucleic acid-free αMEM medium (GIBCO BRL)), and the suspension was disseminated into a 96-well plate (CORNING), for selective culture for about 10 days. Then, the amount of integrin β1 • IgG heavy chain chimeric protein produced in the culture supernatant was determined according to the ELISA method (described later), and the clone showing the highest production was stabilized by cloning according to the limiting dilution method.

[0058] Then, into the stabilized integrin β1 • IgG heavy chain chimeric protein producing CHO cells, the integrin α4 • IgG heavy chain chimeric protein expression vector was transfected according to the lipofectin method as described before. That is, integrin α4 • IgGSRα and pSV2neo (BRL) were mixed at 10 : 1, and the mixture was mixed with lipofectin reagent. The mixture was added dropwise into the cells. After 18 hours of dropwise addition, the mixture was cultured in the said first selective medium for about 2 hours, and the cells were dispersed by trypsin-EDTA treatment. The cells were suspended in a second selective medium (nucleic acid-free αMEM medium (GIBCO BRL) containing 10% FBS (GIBCO) and 1 mg/ml neomycin (GIBCO)), and on a 96-well plate (CORNING), resistant cells were selectively cultured for about 10 days. The amount of integrin α4 • IgG heavy chain chimeric protein and the amount of integrin β1 • IgG heavy chain chimeric protein produced in the culture supernatant were determined according to the ELISA method (described later), and a clone which produced both the chimeric proteins by almost the sane amounts was picked up. The clone was cloned twice according to the limiting dilution method, to be stabilized as a clone capable of producing α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex.

Example 5

Determination of produced integrin α4 • IgG heavy chain chimeric protein and integrin β1 • IgG heavy chain chimeric protein by the ELISA method

[0059] Fifty microliter per well of anti-human integrin α4 antibody (Becton & Dickinson, Clone L25.3) or anti-human integrin β1 antibody (Coulter, Clone 4B4) (12 μg/ml each) was put into a 96-well immunoplate (NUNC), and allowed to stand at 4°C for 16 hours. Then, each well was washed by Dulbecco's phosphate buffered saline (Nissui Seiyaku, not containing Ca or Mg ions, hereinafter called PBS(-)) twice, and non-specific reaction was blocked by PBS(-) containing 25% Block Ace (Snow Brand Milk Products Co., Ltd.). After blocking, the culture supernatant of CHO cells grown in selective medium was properly diluted, and reacted with the coated antibody at room temperature for 1 hour. After the reaction, the surface of the plate was washed with 0.02% Tween-containing PBS(-) (hereinafter called T-PBS) twice. It was then caused to react with biotinated anti-human IgG antibody (Vector) for 1 hour, and the reaction mixture was washed with T-PBS twice, and in succession caused to react with avidin-horseradish peroxidase (Sigma) for 1 hour. The reaction mixture was washed with PBS(-) twice. The PBS(-) was perfectly aspirated, and or thophenylenediamine was used as a substrate for color development. The absorbance at 490 nm were measured using a microplate reader (Bio-rad NOVAPATH), and the clone showing a high absorbance value was selected.

Example 6

Purification of α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex

(1) Culture of CHO cells and preparation of cultured supernatant solution

[0060] The CHO cells highly capable of producing the α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex were cultued in nucleic acid-free αMEM medium containing 5% FBS (Ultra-low IgG grade, GIBCO) (hereinafter called αMEM(-) medium, GIBCO BRL) for one day, to reach semiconfluent, and they were cultured in αMEM(-) medium containing 1% FBS (Ultra-low IgG grade) for 3 days, and the culture supernatant was collected. It was concentrated to 1/10 volume by ultrafiltration using Prep-scale (Millipore), and 1M Hepes solution (pH 8.0) was added to achieve a final concentration of 5 mM, for preparing a starting solution for further purification.

(2) Protein A column chromatography

[0061] The starting solution for further purification was passed through Prosep Guard column (bioPROCESSING), and applied to Prosep A column (bioPROCESSING). After completion of application, it was washed with 10 times the column volume of PBS(-), and the proteins were eluted at a pH 6 - 3 gradient of 0.1M citrate buffer solutions. The peak fraction eluted at pH 3 was collected, and 1M Tris-HCl solution (pH 8.5) was added by 0.1 volume for neutralization. The solution was dialyzed against PBS(-).

(3) Affinity column chromatography

[0062] FMP activated Cellulofine (Seikagaku Kogyo) was equilibrated by a coupling buffer (50 mM Na_2CO_3-$NaHCO_3$ pH 8.5), and a peptide showing sequence No. 3 (hereinafter called CS-1 peptide) synthesized by a peptide synthesizer was added. The mixture was inverted and mixed at 4 °C for 16 hours.

Cys Leu His Gly Pro Glu Ile Leu Asp Val Pro Ser Thr (Sequence No. 3)

After completion of mixing, the mixture was washed with the coupling buffer, and a blocking buffer (0.1 mM monoethanolamine, 50 mM Tris-HCl, pH 8.0) was added. The mixture was inverted and mixed further at room temperature for 6 hours. Then, the mixture was suffificiently washed with TBS solution (150 mM NaCl, 20 mM Tris-HCl, 1 mM $MnCl_2$, pH 7.5), to prepare CS-1 peptide bound Cellulofine column. To the column the starting solution for further purification was applied and allowed to stand at room temperature for 3 hours, and washed with 10 times the column volume of a washing buffer (1M NaCl, 0.1% Triton, 20 mM Tris-HCl, 1 mM $MnCl_2$, pH 7.5) and the same volume of the TBS solution. After completion of washing, an elution buffer (10 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 7.5) was used, to elute the proteins bound to the CS-1 column. The eluate was collected and dialyzed against PBS(-).

(4) SDS-PAGE

[0063] The eluted fractions of (3) were subjected to SDS-PAGE under non-reducing or reducing condition using 6.0 or 7.0% acrylamide gel, and the gel was stained with Coomassie-blue. As a result, under non-reducing condition, two bands considered to be attributable to the α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex and its polymer were observed. Under reducing condition, two bands (170 kDa and 135 kDa) considered to be attributable to the integrin α4 • IgG heavy chain chimeric protein and the integrin β1 • IgG heavy chain chimeric protein and two bands (80 kDa and 90 kDa) considered to be attributable to the intramolecular cleavage of the integrin α4 • IgG heavy chain chimeric protein (Hemler, M. E. et al., J. Biol. Chem., 262, 11478-11485 (1987)) were observed. These results suggest that the eluted protein of (3) has a molecular structure considered to be α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex, and that the molecules constituting the heterodimer are linked by a disulfide bond between the IgG heavy chains.

Example 7

Identification of α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex, and examination of its structural stability

(1) Immunoprecipitation using anti-integrin antibodies and influence of a cationic chelating agent

[0064] The basic method conformed to a published book ("Antibodies", Harlow, E. et al., (1988), Cold Spring Harbor Lab. Press, New York). That is, the eluted ptotein of Example 6 (3) considered to be α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex was ^{125}I-labeled using the lactoperoxidase method. Then, Affigel-10 (Bio-rad) was washed with 0.1 M Hepes solution (pH 8.0), and normal murine IgG, anti-human integrin α4 antibody (clone 11C2B) and anti-human integrin β1 antibody (clone 4B4) were added. Reaction was effected at 4°C for 16 hours to cause covalent bonding, to prepare normal murine IgG beads and the respective antibody beads. Then, the ^{125}I labeled α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex and normal murine IgG beads were inverted and mixed at 4 °C for 4 hours for preclearing, and the mixture and the antibody beads were inverted and mixed at 4°C for 16 hours. After completion of mixing, the beads were washed with a washing buffer (200 mM Tris-HCl, 0.5 M NaCl, 0.1% NP-40, 1 mM $MgCl_2$ or 10 mM EDTA, pH 8.0) three times. After completion of washing, a sample buffer for electrophoresis was added to the beads for treatment at 100 °C for 5 minutes, and the mixture was centrifuged. The supernatant solution was analysed by electrophoresis under reducing condition. After completion of electrophoresis, the gel was dried by a gel dryer, and the protein was detected by autoradiography.

[0065] As a result of immunoprecipitation in the presence of 1 mM $MgCl_2$, from the beads of both the anti-human integrin α4 antibody and the anti-human integrin β1 antibody, the same precipitation patterns expected from the structure of α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex were obtained. Thus, the protein obtained in (3) of Example 6 was identified as α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex.

[0066] On the other hand, the immunoprecipitation pattern obtained by using the anti-integrin β1 antibody beads in the presence of 10 mM EDTA was the same as that in the presence of 1 mM $MgCl_2$, to clarify that the association between integrin α4 • IgG heavy chain chimeric protein and integrin β1 • IgG heavy chain chimeric protein does not depend on cations. The above results suggest that the eluted protein obtained in (3) of Example 6 was certain α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex, and if the result of (4) of Example 6 is also taken into account, it is strongly suggested that the association between both the proteins is stable association through a disulfide bond existing the IgG heavy chains.

(2) Examination on the structural stability of α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex by sequential immunoprecipitation

[0067] According to (1), ^{125}I labeled α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex was caused to react with normal murine IgG beads, anti-human integrin α4 antibody (11C2B) beads or anti-human integrin β1 antibody (4B4) beads at 4°C for 4 hours, and the reaction mixture was washed. After washing, the reaction mixture was boiled at 100°C for 5 minutes in the presence of 2% SDS and centrifuged, and the supernatant (primary immunoprecipitation sample) was diluted to 10 times by 1% BSA-containing PBS, and was again reacted with the anti-integrin β1 antibody beads and the anti-integrin α4 antibody beads at 4°C for 16 hours. After completion of reaction, the beads were washed, and a sample buffer for electrophoresis was added. The mixture was treated at 100°C for 5 minutes and centrifuged. The supernatant solution (secondary immunoprecipitation sample) was analyzed by SDS-PAGE/autoradiography.

[0068] As a result, the electrophoretic pattern obtained by the primary immunoprecipitation was also similarly observed in the secondary immunoprecipitation. This result suggests that the association between the α4 • IgG heavy chain chimeric protein and the β1 • IgG heavy chain chimeric protein in the α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex is not dissociated either by boiling in the presence of 2% SDS, and strongly supports that the complex has a stable heterodimer structure based on a disulfide bond.

Example 8

Binding of α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex to VCAM-1

[0069] It was examined that the α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex produced by CHO cells can be bound to the ligand of integrin α4β1 by using the cells expressing VCAM-1. Human normal umbilical intravenous endothelial cells were cultured with IL-1 3U/ml for 16 hours, to prepare VCAM-1 expressing cells. The cells were treated by 1 mM EDTA at 37°C for 15 minutes, for dispersion as single cells. The cells (2 x 10^5 cells per sample tube) were cultured with the supernatant of the CHO cells producing α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex for 30 minutes in the presence of 1 mM (final concentration) $MnCl_2$ or 3 mM (final concentration) EDTA. After completion of reaction, the cells were washed twice by centrifugation at 1200 rpm at room temperature for 5 minutes using a buffer for binding assay (24 mM Tris-CHI, 10 mM Hepes, 150 mM NaCl, 1 mM MnCl $_2$ or 1 mM EDTA, 1% BSA, 2 mM glucose, pH 7.4). After washing, FITC labeled-anti-human IgG antibody (Cappel) was added, and incubated at room temperature for 20 minutes. The cells were washed by the same buffer, and the chimeric proteins bound to the cells were determined by a flow cytometer (ELITE, Coulter).

[0070] The results are shown in Fig. 1. It was observed that the fluorescence intensity showing the binding of α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex increased by culturing the VCAM-1 expression cells with the supernatant containing α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex. The binding was inhibited by adding anti-human integrin antibodies (anti-α • antibody: clone L25.3, 10 μm/ml + anti-β1 antibody: clone 4B4, 10 μm/ml) or 3 mM EDTA. This result suggests that the α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex can be bound to VCAM-1 like the integrin α4β1 existing on the surfaces of cell membranes, and furthermore that the binding is α4β1-specific and retains a feature of the binding that it is dependent on cations.

Example 9

Binding of α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex to a peptide fragment of fibronectin

[0071] The capability of α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex to be bound to the peptide fragment (sequence No. 3) of the other ligand, fibronectin was also examined.

[0072] At first, according to the said report (Humphries, M. J. et al., J. Biol. Chem., 262, 6886-6892 (1987)), the peptide fragment of sequence No. 3 (CS-1 peptide) was bound to rabbit IgG (Sigma), to prepare CS-1-IgG. The CS-1-IgG was diluted by PBS(-), and put in a 96-well immunoplate (NUNC) by 100 μl/well, and allowed to stand at 4°C for 16 hours, to be formed as a solid phase on the plate.

[0073] After completion of standing, the surface of the plate was washed with PBS(-) twice and treated with denatured 1% BSA(heat-natured at 80°C for 10 minutes)-PBS solution (300 μl/well) at 4°C for 3 hours to block the nonspecific reaction. Then, the solid phase CS-1-IgG and the CHO culture supernatant (100 μl) containing α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex were reacted with each other at 30°C for 3 hours. The non-bound α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex was removed by washing with 0.1% BSA-containing TBS buffer (150 mM NaCl, 25 mM Tris-HCl, 1 mM $MnCl_2$, pH 7.4) twice, and the bound α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex was detected by biotin labeled anti-human IgG antibody (Vector) as the primary antibody and avidin labeled horseradish peroxidase (Sigma) as the secondary antibody. The surface of the plate was washed with the TBS buffer. Orthophenylenediamine was added as a substrate to it for color development, and the absorbance at 490 nm were measured.

[0074] The results are shown in Fig. 2. The react ion with α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex showed a rise in the absorbance indicating the binding to CS-1 peptide. The binding was almost perfectly inhibited by the presence of anti-integrin αA antibody (clone L25.3), anti-integrin β1 antibody (clone 4B4) or 5 mM EDTA. Therefore, it was clarified that α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex can be bound also to the CS-1 peptide which is a peptide fragment of fibronectin, and that a feature of binding that it depends on cations is retained.

Example 10

Evaluation of an inhibitory peptide by using a system for determining the binding of α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex to a peptide fragment of fibronectin

[0075] In the binding determination system of Example 9, the effects of three peptides, i.e., sequence No. 16 (hereinafter called GPEILDVPST), 17 (hereinafter called GPEILEVPST) and 18 (hereinafter called GRGDSP) were examined.

Gly Pro Glu Ile Leu Asp Val Pro Ser Thr (Sequence No. 16)

Gly Pro Glu Ile Leu Glu Val Pro Ser Thr (Sequence No. 17)

Gly Arg Gly Asp Ser Pro (Sequence No. 18)

[0076] The all peptides were synthesized by a peptide synthesizer. The peptide and 100 μl of CHO cultured supernatant solution containing αα4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex were mixed at room temperature for 20 minutes, and the binding to CS-1-IgG was determined according to the method in Example 9. The results are shown in Fig. 3. GPEILDVPST showed temperature-dependent inhibitory activity in a range of 0.1 to 10 μmg/ml, but GPEILEVPST and GRGDSP did not show any inhibition of the binding. These results show that the binding determination system in Example 9 allows to detect the inhibiting effect of the peptide (GPEILDVPST) inhibiting the binding between integrin α4β1 and CS-1 peptide specifically .

Example 11

Construction of integrin α2 • IgG heavy chain chimeric protein expression vector

[0077] The DNA fragment coding for the extracellular portion of integrin α2 was divided into α2-1 and α2-2 based on the reported cDNA sequence information (Takada, Y. et al., J. Cell. Biol., 109, 397-407 (1989)) and subcloned, and they were integrated on an expression vector. At first, the RNA of human fibroblast cell line MRC-5 (ATCC CCL 171) as integrin α2 expressing cell was separated, and an oligo dT cellulose column was used to purify PolyA(+)RNA. Based on it, a single stranged cDNA was synthesized and used as the template of PCR. As PCR primers, DNA oligomers of sequence Nos. 20 and 21 were synthesized for α2-1, and DNA oligomers of sequence Nos. 22 and 23, for α2-2.

5' -GCTCGAGCAAACCCAGCGCAACTACGC-3' (Sequence No. 20)

5' -ATACTGCCCTGATGACCATTG-3' (Sequence No. 21)

5' -GATGGCTTTAATGATGTGATTG-3' (Sequence No. 22)

5' -TGTTGGTACTTCGGCTTTCTC-3' (Sequence No. 23)

[0078] The template cDNA, primers, dNTPs and Taq polymerase were mixed in a PCR buffer and PCR was performed 30 cycles by a thermal cycler (reaction conditions: 94°C 1 minute - 60°C 2 minutes - 72°C 3 minutes). The amplified DNA fragment of α2-1 was digsted by restriction enzymes Xho I and EcoR I , and the DNA fragment of α2-2 was blunted at the termini by T4DNA polymerase treatment and digested by restriction enzyme EcoR I. Each fragment was purified. The two purified DNA fragments were caused to react in a phosphating reaction solution (50 mM Tris-HCl, 10 mM $MgCl_2$, 25 mM DTT, 1 mM ATP, 0.1 U/μl T4 polynucleotide kinase (Takara), pH 8.0) at 37 °C for 1 hour, and the reaction mixture was heat-treated at 68°C for 5 minutes to inactivate the enzyme. Then, $IgG_1SR\alpha$ prepared in Example 1 was digested by restriction enzyme BamH I and caused to react in Klenow reaction solution (66 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 0.2 mM dNTPs, 0.05 U/ μl Klenow fragment (Takara), pH 7.5) at 37°C for 30 minutes, to blunt the

termini, and the reaction mixture was heat-treated at 70°C for 5 minutes to inactivate the enzyme. Furthermore, a large DNA fragment was digested by restriction enzyme Xho I, and pudified. The two (α2-1 and α2-2) DNA fragments phosphated before were inserted into the large DNA fragment, to obtain a plasmid DNA. The obtained base sequence coding for integrin α2 • IgG heavy chain chimeric protein is shown in sequence No. 19. This plasmid (integrin α2 • IgGSRα) is hereinafter called α2 • IgG heavy chain chimeric protein expression vector.

Example 12

Transfection of integrin α2 • IgG heavy chain chimeric protein expression vector and integrin β1 • IgG heavy chain chimeric protein expression vector into animal cells, and their manifestation

[0079] The integrin α2 • IgG heavy chain chimeric protein expression vector was transfected into the integrin β1 • IgG heavy chain chimeric protein producing CHO cells prepared and stabilized in Example 4, according to the lipofectin method described in Example 4. That is, integrin α2 • IgGSRα and pSV2neo (BRL) were mixed at 10 : 1, and the mixture was mixed with lipofectin reagent. The mixture was added dropwise to the cells. Eighteen hours after completion of dropwise addition , the mixture was cultured in a first selective medium for 2 days, and the cells were dispersed by trypsin-EDTA treatment. The cells were suspended in a second selective medium, and the suspension was disseminated into a 96-well plate. Resistant cells were selectively cultured for about 10 days. Then, the amount of integrin α2 • IgG heavy chain chimeric protein and the amount of integrin β1 • IgG heavy chain chimeric protein produced in the culture supernatant were determined according to the ELISA method (described later), and a clone producing almost the same amounts of both the chimeric proteins was picked up. The clone was cloned twice according to the limiting dilution analysis, to be stabilized as a clone capable of producing α2 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex.

Example 13

Determination of the amounts of integrin α2 • IgG heavy chain chimeric protein and integrin β1 • IgG heavy chain chimeric protein by the ELISA method

[0080] Fifty microliter per well of anti-human integrin α2 antibody (Becton & Dickinson, clone P1E6) or anti-human integrin β1 antibody (clone 4B4) (2 μg/ml each) was put into a 96-well immunoplate , and allowed to stand at 4°C for 16 hours. Then, each well was washed with PBS(-) twice, blocked, and the culture supernatant of the CHO cells grown in second selective medium was properly diluted and reacted with the coated-antibody at room temperature for 1 hour. After the reaction, the surface of the plate was washed with T-PBS twice, and caused to react with biotinated anti-human IgG antibody for 1 hour and with avidin-horseradish peroxidase for 1 hour, and the reaction mixture was washed with PBS(-) twice. After completion of reaction, orthophenylenediamine was used as a substrate for color development, and the absorbance values at 490 nm were measured using a microplate reader. A clone showing a high absorbance value was selected.

Example 14

Purification of α2 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex

(1) Culture of CHO cells and preparation of cultured supernatant solution

[0081] The CHO cells highly capable of producing α2 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex were cultured in an αMEM(-) medium containing 5% FBS (Ultra-low IgG grade) for 1 day, to reach semiconfluent, and they were cultured on an αMEM(-) medium containing 1%FBS (Ultra-low IgG grade) for 3 days. The culture supernatant was collected, and concentrated to 1/10 volume by ultrafiltration. Then, 1M Hepes solution (pH 8.0) was added to achieve a final concentration of 5 mM, to obtain a starting solution for further purification.

(2) Protein A colum chromatography

[0082] The starting solution for further purification was passed through Prosep Guard column, and applied to Prosep A column. After completion of application, it was washed with 10 times the column volume of PBS (-), and in succession, the proteins were eluted at a pH 6 to 3 gradient of 0.1M citrate buffers. The peak fraction eluted at pH 3 was collected, and 1M Tris-HCl solution (pH 8.5) was added by 0.1 volume for neutralization. The mixture was dialyzed against PBS(-).

(3) Affinity column chromatography

[0083] According to a report (Kirchhofer, D. et al., J. Biol. Chem., 265, 615-618 (1990)), a collagen immobilized column with a collagen (TypeI, Sigma) coupled to cyanogen-bromide-activated Sepharose (Sigma) was prepared. Then, the starting solution for further purification was equilibrated in a TBS buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, pH 7.5), applied to a column, allowed to stand at room temperature for 3 hours, and washed with 10 times the column volume of a washing buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 100 mM octyl glucopyranoside, pH 7.5). After completion of washing, an elution buffer (20 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl, 50 mM octyl glucopyranoside, pH 7.5) was used to elute the protein bound to the column. The eluate was collected and dialyzed against PBS(-).

(4) SDS-PAGE

[0084] The eluted fraction of (3) was subjected to SDS-PAGE using 7.0% acrylamide gel under non-reducing or under reudcing condition, and the gel was stained with Coummassie-blue. As a result, a band considered to be attributable to $\alpha 2$ • IgG heavy chain-$\beta 1$ • IgG heavy chain chimeric protein heterodimer complex was observed. Under reducing condition, two bands (185 kDa and 135 kDa) considered to be attributable to integrin $\alpha 2$ • IgG heavy chain chimeric protein and integrin $\beta 1$ • IgG heavy chain chimeric protein were observed. These results suggest that the eluted protein has a molecular structure considered to be $\alpha\alpha 2$ • IgG heavy chain-$\beta 1$ • IgG heavy chain chimeric protein heterodimer complex, and is linked by a disulfide bond between the IgG heavy chains.

Example 15

Identification of $\alpha 2$ • IgG heavy chain-$\beta 1$ • IgG heavy chain chimeric protein heterodimer complex, and examination of its structural stability

[0085] The eluted protein of (3) of Example 14 was ^{125}I-labeled, and subjected to immunoprecipitation using the beads coupled with normal murine IgG, anti-human integrin $\alpha 2$ antibody (clone P1E6) or anti-human integrin $\beta 1$ antibody (clone 4B4) as described in Example 7, and to SDS-PAGE/autoradiography under reducing condition.

[0086] As a result, in both 1 mM $MgCl_2$ and 10 mM EDTA, from the beads of both anti-human integrin $\alpha 2$ antibody and anti-human integrin $\beta 1$ antibody, the same precipitation patterns expected from the structure of $\alpha 2$ • IgG heavy chain-$\beta 1$ • IgG heavy chain chimeric protein heterodimer complex could be obtained. These results show that the eluted protein obtained in (3) of Example 14 is certainly $\alpha 2$ • IgG heavy chain-$\beta 1$ • IgG heavy chain chimeric protein heterodimer complex, and with the results of (4) of Example 14 also taken into account, it is strongly suggested that the association of both the proteins is stable through a disulfide bond existing the IgG heavy chains.

Example 16

Examination on the capability of $\alpha 2$ • IgG heavy chain-$\beta 1$ • IgG heavy chain chimeric protein heterodimer complex to be bound to a collagen, and its specificity

[0087] The capability of $\alpha 2$ • IgG heavy chain-$\beta 1$ • IgG heavy chain chimeric protein heterodimer complex to be bound to a collagen which is a ligand of integrin $\alpha 2\beta 1$ was examined.

[0088] At first, a collagen (Cell Matrix Type 3 mg/ml) was diluted to 0.1 µg/ml by 0.02M acetic acid solution, and put in an immunoplate by 100 µl/well, being kept at 4°C for 16 hours. Then, the collagen solution was removed by suction, and the plate was washed with PBS(-) twice for neutralization. Heat-denaturated 1% BSA-PBS solution was put in the plate by 300 µl/well for blocking at room temperature for 3 hours. After completion of blocking, it was rinsed With PBS (-) twice, to prepare a collagen coated plate.

[0089] The cultured supernatant of CHO (100 µl) containing $\alpha 2$ • IgG heavy chain-$\beta 1$ • IgG heavy chain chimeric protein heterodimer complex was reacted at 30°C for 3 hours. After completion of reaction, as described in Example 9, the amount of bound $\alpha 2$ • IgG heavy chain-$\beta 1$ • IgG heavy chain chimeric protein heterodimer complex was determined.

[0090] As a result, as shown in Fig. 4, the absorbance showing the binding of $\alpha 2$ • IgG heavy chain-$\beta 1$ • IgG heavy chain chimeric protein heterodimer complex to the collagen was increased. The binding was almost perfectly inhibited in the coexistence of 10 µg/ml of anti-human integrin $\alpha 2$ antibody (clone P1E6) and anti-human integrin $\beta 1$ antibody (clone 4B4), or in the presence of 5 mM EDTA respectively. This result shows that $\alpha 2$ • IgG heavy chain-$\beta 1$ • IgG heavy chain chimeric protein heterodimer complex can be bound to a collagen like the integrin $\alpha 2\beta 1$ existing on the surfaces of cell membranes, and furthermore that the binding is $\alpha 2\beta 1$-specific and that the feature of the binding that it depends on cations is retained.

Example 17

Acquisition of a peptide capable of being bound to α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex, and evaluation of its inhibitory activity

[0091] At first, α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex purified in Example 6, or human IgG was prepared at a proper concentration by PBS (-) and was coated on a plastic plate at 4°C for 16 hours, being formed as a solid phase on a plastic plate. Then, according to a report (Cott, J. K. and Smoth, G. P., Science, 249, 386-390 (1990)), a phage peptide library in which a random six amino acid residues were cyclyzed by the disulfide bond of cysteine at both the ends was prepared and suspended in 0.1% BSA-containing TBS buffer. The phage peptide library was reacted with human IgG at 30°C for 3 hours, to absorb phage peptides capable of being bound to IgG. Then, the non-absorbed phases were reacted with α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex at 30°C for 3 hours, and the reaction mixture was washed with 0.1% BSA-containing TBS buffer twice to remove the phage peptides incapable of being bound to the heterodimer complex. Only the phage peptides capable of being bound were collected after elution with 0.1M glycine-hydrochloric acid (pH 2.2). After collection, the phage was amplified and the above mentioned binding operation was repeated further twice. The only the phage peptides capable of being bound to the heterodimer complex were selectively concentrated. In the final elution operation, phage peptides capable of being bound to the heterodimer complex were eluted using 10 mM EDTA and 0.1M glycine-hydrochloric acid in two steps, and the amino acid sequences of the respective peptides were analyzed. Of them, eight sequences (sequence Nos. 24 to 31) are shown in Table 1. Furthermore, they were examined using the binding assay system of Example 9, and the IC50 values of the four peptide sequences showing binding inhibitory activity are shown in Table 1.

Table 1

Elution condition	Sequence								Inhibitory activity IC50 (μM)	Sequence No.
EDTA	Cys*	Ile	Pro	Glu	Leu	Ile	Val	Cys*	1.2	24
	Cys*	Met	Arg	Tyr	Thr	Ser	Ala	Cys*	2.3	25
	Cys*	Glu	Trp	Met	Lys	Arg	Phe	Cys*		26
	Cys*	Tyr	Thr	Thr	Arg	Leu	Lys	Cys*		27
Glycine-hydrochloric acid	Cys*	Leu	Arg	Tyr	Ser	Val	Pro	Cys*	1.8	28
	Cys*	Ile	Val	Asn	Arg	Leu	Gly	Cys*		29
	Cys*	Gly	Leu	Gln	Ala	Leu	Pro	Cys*	10	30
	Cys*	Lys	Leu	Lys	Gly	Thr	Met	Cys*		31

Cys* indicates a disulfide bond.

Example 18

Acquisition of a low weight molecular compound capable of inhibiting the binding between the peptide fragment on fibronectin and α4 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex

[0092] Reagents and reported compounds were picked up at random, adjusted to a final concentration of 50 or 100 μg/ml, and added to the binding determination system in Example 9. Compounds showing inhibitory activity were obtained. Of the obtained compounds, the binding inhibitory activities of the four compounds of Norethynodrel (Sigma), D-Penicillamine (Aldrich, Weigert, W. M. et al., Angew. Chem. Int. Ed. Eng., 14, 330-336 (1975), γ-2-Naphthyl butyric acid (Fieser, L. F. J. Am. Chem. Soc., 70, 3197-3203 (1948)), 1-Adamantaneacetic acid (Aldrich) were shown in Table 2.

Table 2

Name of compound	Concentration (μg/ml)	Inhibition rate (%)
Norethynodrel	50	28
D-Penicillamine	50	51
γ-2-Naphtyl butyric acid	100	37
1-Adamantaneacetic acid	100	65

Example 19

Preparation of α2 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex liposome

[0093] A liposome was prepared according to the Martin et al.'s method (Martin, F. J. et al., Biochemistry, 20, 4229, (1981)). At first, an activated SH group was introduced into dipalmitoyl phosphatidyl ethanolamine (DPPE, Sigma) using di-crosslinking reagent N-succineimidyl 3-(2-pyridyldithio)propionate (SDPD, Sigma), to prepare pyridylthiopropionyl dipalmitoyl phosphatidyl ethanolamine (PDP-DPPE). The PDP-DPPE, dipalmitoyl phosphatidyl choline (DPPC) and cholesterol were mixed, to prepare a lipid film, and it was treated by a sonicator. Then, a filter was used to obtain a liposome uniform in diameter(PDP-DPPE liposome). Then, α2 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex or human IgG (Cappel) used as a negative control were dissolved in a Hepes buffer (100 mM Hepes, 150 mM NaCl, pH 8.0), and SDPD was added for reaction for 30 minutes. The reaction solution was applied to PD-10 column (Pharmacia), and eluted by 0.1M acetic acid-sodium acetate buffer (pH 5.5). To the eluate, dithiothreitol was added for treatment for 20 minutes, and the mixture was applied to PD-10 column again and eluted by a Hepes buffer (100 mM Hepes, 150 mM NaCl, pH 8.0), to obtain SDPD coupled α2 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex. The SDPD modified heterodimer complex and the PDP-DPPE liposome were caused to react with each other at room temperature for 24 hours, and the reaction mixture was separated by Sepharose 4B column (Sigma). From the peak fraction, α2 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex liposome was obtained.

[0094] The amount of α2 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex bound on the liposome was determined by a densitometer (ATTO) after SDS-PAGE/Coummassie staining, and adjusted to final concentration of 1 mg/ml.

Example 20

Flow cytometry of α2 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex liposome

[0095] α2 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex liposome was dispersed in 1 mM EDTA-containing PBS(-), and caused to react with anti-human integrin α2 antibody (clone P1E6) or anti-human integrin β1 antibody (clone 4B4) at room temperature for 30 minutes. After completion of reaction, the reaction mixture was centrifuged at 15000 rpm for 10 minutes, being followed by washing with 1 mM EDTA-containing PBS(-) and suspended into the solution again. Into the suspension, FITC labeled anti-murine IgG antibody (Cappel, 10 μg/ml) was added as a secondary antibody, and reacted at room temperature for 30 minutes. After completion of reaction, the reac-

tion mixture was similarly washed by centrifugation, and flow cytometry analysis (ELITE, Coulter) was performed.

[0096] As a result, the positive reactions for both the antibodies were detected, confirming that α2 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex was bound on the liposome.

Example 21

Binding activity of α2 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex to a collagen

[0097] A collagen (Cell Matrix Type, 3 mg/ml) was diluted by 0.02M acetic acid solution, and the solution was put in an immunoplate by 100 μl/well, being kept at 4°C for 16 hours. Then, the collagen solution was removed by suction, and the plate was washed with PBS(-) twice for neutralization, and heat-denaturated 1% BSA-PBS solution was put in the plate by 300 μl/well for blocking at room temperature for 3 hours. After completion of blocking, the plate was rinsed with PBS(-) twice, to prepare a collagen coated plate.

[0098] Normal human plasma (George King) and von Willebrand's factor deficient (severe) plasma (George Kind) were treated with anti-human IgG antibody and protein A, and dialyzed against PBS(-) for 24 hours, to remove the contained sodium citrate. In order that the Ca ion and Mg ion concentration might be a physiological concentration in the blood when used, $CaCl_2$ and $MgCl_2$ were added to achieve final concentrations of 1.2 mM and 0.2 mM respectively. Into the normal human plasma and von Willebrand's factor deficient plasma adjusted in cation concentration, α2 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex liposome or human IgG liposome was suspended to achieve protein concentrations of 1 to 100 ng/ml. Any of the suspensions was put in the collagen coated plate by 100 μl/well. The plate was shaken by a plate shaker at 100 rpm, for react ion at room temperature for 15 minutes. After completion of reaction, the non-bound liposome was removed by washing with a PB solution (1.2 mM $CaCl_2$, 0.2 mM $MgCl_2$, 1% BSA-containing PBS, pH 7.4), and the bound liposome was immobilized by 1% glutaraldehyde-PBS at room temperature for 30 minutes. After completion of immobilization, a heat-denaturated BSA-PBS solution was used for blocking at room temperature for 1 hour. Then, as described in Example 16, it was caused to react with biotin labeled human IgG antibody used as a primary antibody and avidin labeled horseradish peroxidase used as a secondary antibody, and washed with a TBS buffer. Into it, orthophenylenediamine was added as a substrate for color development, and the absorbance at 490 nm were measured. To examine the effect of 5 mM EDTA, anti-integrin α2 antibody (clone P1E6, 10 μg/ml) and anti-integrin β1 antibody(clone 4B4, 10 μg/ml), it was caused to react with the liposome suspension at room temperature for 15 minutes before reaction with the collagen.

[0099] The results are shown in Figs. 5 and 6. In the normal human plasma, the human IgG liposome as a negative control was not found to be bound to the collagen, but the binding of α2 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex liposome to the collagen was increased with the concentration dependent on manner. Also when the von Willebrand's factor deficient plasma was used, equivalent binding was detected. Furthermore, the binding to the collagen observed when 30 ng/ml of α2 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex liposome was added to the normal plasma was completely inhibited by adding EDTA as a cation chelating agent or the antibodies. The results show that in plasma with a physiological cation concentrtion, α2 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex liposome is bound to a collagen like platelets, and strongly suggest that it can be a substitute of adhesive platelets, and can be a reagent for monitoring the collagen exposed region. Furthermore, it is indicated that since equivalent binding activity was shown also in von Willebrand's factor defincient plasma, the liposome can also be used in the plasma with coagulation abnormality such as von Willebrand's disease.

Example 22

Analysis of collagen covering state by α2 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex liposome

[0100] Five microliters of a collagen solution was spotted at the center of each of the wells of a Lab-Tek chamber slide (Intermed, 8-well type, plastic) and allowed to stand for 16 hours, then washed and treated for blocking. Then, a suspension in which α2 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex liposome was suspended in normal human plasma to achieve a protein concentration of 30 ng/ml as described in Example 21 was put in the slide by 200 μl/well, for reaction under the same conditions. After completion of reaction, the non-bound liposome was removed by washing with a PB buffer, and the retained was immobilized and treated for blocking. Then, it was bound to biotin labeled anti-human IgG antibody as a primary antibody and with avidin labeled horseradish peroxidase as a secondary antibody, and was washed with a TBS buffer. After completion of washing, diaminobenzidine was added for staining, to observe the covering state of the α2 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex liposome bound on the collagen.

[0101] With the human IgG liposome, the collagen coated portion was not stained, but with α2 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex liposome, the collagen coated portion was entirely stained. Therefore, since the α2 • IgG heavy chain-β1 • IgG heavy chain chimeric protein heterodimer complex liposome covered the collagen coated portion only, it was strongly suggested that the liposome could be a substitute of adhesive platelets.

Industrial Availability:

[0102] The present invention provides integrin-immunoglobulin chimeric protein heterodimer complexes in which the α chain and the β chain of an integrin are stably associated. The obtained integrin-immunoglobulin chimeric protein heterodimer complexes can be directly used as drugs, and can also be used for determining the binding between an integrin and a ligand, and searching for a substance capable of being bound to an integrin and a substance inhibiting the binding between an integrin and a ligand. They can also be used as diagnostic reagents.
[0103] Furthermore, among the heterodimer comlexes, especially integrin α2β1-immunoglobulin chimeric protein heterodimer complex can be used as a substitute of platelets. Furthermore, integrin α2β1-immunoglobulin chimeric protein heterodimer complex can be used as a therapeutic or preventive agent for bleeding tendency involved in thrombocytopenia, platelet function abnormality, etc. Furthermore, it can also be used as a reagent for monitoring the exposed region of an extracellular matrix and for the targeting therapy.

Sequence Table

Sequence No. 1

Length of sequence: 4228

Type of sequence: Nucleic acid

Sequence

ATG TTC CCC ACC GAG AGC GCA TGG CTT GGG AAG CGA GGC GCG AAC CCG 48
Met Phe Pro Thr Glu Ser Ala Trp Leu Gly Lys Arg Gly Ala Asn Pro
-35 -30 -25

GGC CCC GAA GCT GCA CTC CGG GAG ACG GTG ATG CTG TTG CTG TGC CTG 96
Gly Pro Glu Ala Ala Leu Arg Glu Thr Val Met Leu Leu Leu Cys Leu
-20 -15 -10

GGG GTC CCG ACC GGC AGG CCT TAC AAC GTG GAC ACT GAG AGC GCG CTG 144
Gly Val Pro Thr Gly Arg Pro Tyr Asn Val Asp Thr Glu Ser Ala Leu
-5 1 5

CTT TAC CAG GGC CCC CAC AAC ACG CTG TTC GGC TAC TCG GTC GTG CTG 192
Leu Tyr Gln Gly Pro His Asn Thr Leu Phe Gly Tyr Ser Val Val Leu
10 15 20 25

CAC AGC CAC GGG GCG AAC CGA TGG CTC CTA GTG GGT GCG CCC ACT GCC 240
His Ser His Gly Ala Asn Arg Trp Leu Leu Val Gly Ala Pro Thr Ala
30 35 40

AAC TGG CTC GCC AAC GCT TCA GTG ATC AAT CCC GGG GCG ATT TAC AGA 288
Asn Trp Leu Ala Asn Ala Ser Val Ile Asn Pro Gly Ala Ile Tyr Arg
45 50 55

TGC AGG ATC GGA AAG AAT CCC GGC CAG ACG TGC GAA CAG CTC CAG CTG 336
Cys Arg Ile Gly Lys Asn Pro Gly Gln Thr Cys Glu Gln Leu Gln Leu
60 65 70

GGT AGC CCT AAT GGA GAA CCT TGT GGA AAG ACT TGT TTG GAA GAG AGA 384
Gly Ser Pro Asn Gly Glu Pro Cys Gly Lys Thr Cys Leu Glu Glu Arg
75 80 85

```
GAC AAT CAG TGG TTG GCG GTC ACA CTT TCC AGA CAG CCA GGA GAA AAT      432
Asp Asn Gln Trp Leu Gly Val Thr Leu Ser Arg Gln Pro Gly Glu Asn
 90                  95                 100                 105
GGA TCC ATC GTG ACT TGT GGG CAT AGA TGG AAA AAT ATA TTT TAC ATA      480
Gly Ser Ile Val Thr Cys Gly His Arg Trp Lys Asn Ile Phe Tyr Ile
                110                 115                 120
AAG AAT GAA AAT AAG CTC CCC ACT GGT GGT TGC TAT GGA GTG CCC CCT      528
Lys Asn Glu Asn Lys Leu Pro Thr Gly Gly Cys Tyr Gly Val Pro Pro
            125                 130                 135
GAT TTA CGA ACA GAA CTG AGT AAA AGA ATA GCT CCG TGT TAT CAA GAT      576
Asp Leu Arg Thr Glu Leu Ser Lys Arg Ile Ala Pro Cys Tyr Gln Asp
        140                 145                 150
TAT GTG AAA AAA TTT GGA GAA AAT TTT GCA TCA TGT CAA GCT GGA ATA      624
Tyr Val Lys Lys Phe Gly Glu Asn Phe Ala Ser Cys Gln Ala Gly Ile
    155                 160                 165
TCC AGT TTT TAC ACA AAG GAT TTA ATT GTG ATG GGG GCC CCA GGA TCA      672
Ser Ser Phe Tyr Thr Lys Asp Leu Ile Val Met Gly Ala Pro Gly Ser
170                 175                 180                 185
TCT TAC TGG ACT GGC TCT CTT TTT GTC TAC AAT ATA ACT ACA AAT AAA      720
Ser Tyr Trp Thr Gly Ser Leu Phe Val Tyr Asn Ile Thr Thr Asn Lys
                190                 195                 200
TAC AAG GCT TTT TTA GAC AAA CAA AAT CAA GTA AAA TTT GGA AGT TAT      768
Tyr Lys Ala Phe Leu Asp Lys Gln Asn Gln Val Lys Phe Gly Ser Tyr
            205                 210                 215
TTA GGA TAT TCA GTC GGA GCT GGT CAT TTT CGG AGC CAG CAT ACT ACC      816
Leu Gly Tyr Ser Val Gly Ala Gly His Phe Arg Ser Gln His Thr Thr
        220                 225                 230
GAA GTA GTC GGA GGA GCT CCT CAA CAT GAG CAG ATT GGT AAG GCA TAT      864
Glu Val Val Gly Gly Ala Pro Gln His Glu Gln Ile Gly Lys Ala Tyr
    235                 240                 245
```

ATA TTC AGC ATT GAT GAA AAA GAA CTA AAT ATC TTA CAT GAA ATG AAA 912
Ile Phe Ser Ile Asp Glu Lys Glu Leu Asn Ile Leu His Glu Met Lys
250 255 260 265
GGT AAA AAG CTT GGA TCG TAC TTT GGA GCT TCT GTC TGT GCT GTG GAC 960
Gly Lys Lys Leu Gly Ser Tyr Phe Gly Ala Ser Val Cys Ala Val Asp
270 275 280
CTC AAT GCA GAT GGC TTC TCA GAT CTG CTC GTG GGA GCA CCC ATG CAG 1008
Leu Asn Ala Asp Gly Phe Ser Asp Leu Leu Val Gly Ala Pro Met Gln
285 290 295
AGC ACC ATC AGA GAG GAA GGA AGA GTG TTT GTG TAC ATC AAC TCT GGC 1056
Ser Thr Ile Arg Glu Glu Gly Arg Val Phe Val Tyr Ile Asn Ser Gly
300 305 310
TCG GGA GCA GTA ATG AAT GCA ATG GAA ACA AAC CTC GTT GGA AGT GAC 1104
Ser Gly Ala Val Met Asn Ala Met Glu Thr Asn Leu Val Gly Ser Asp
315 320 325
AAA TAT GCT GCA AGA TTT GGG GAA TCT ATA GTT AAT CTT GGC GAC ATT 1152
Lys Tyr Ala Ala Arg Phe Gly Glu Ser Ile Val Asn Leu Gly Asp Ile
330 335 340 345
GAC AAT GAT GGC TTT GAA GAT GTT GCT ATC GGA GCT CCA CAA GAA GAT 1200
Asp Asn Asp Gly Phe Glu Asp Val Ala Ile Gly Ala Pro Gln Glu Asp
350 355 360
GAC TTG CAA GGT GCT ATT TAT ATT TAC AAT GGC CGT GCA GAT GGG ATC 1248
Asp Leu Gln Gly Ala Ile Tyr Ile Tyr Asn Gly Arg Ala Asp Gly Ile
365 370 375
TCG TCA ACC TTC TCA CAG AGA ATT GAA GGA CTT CAG ATC AGC AAA TCG 1296
Ser Ser Thr Phe Ser Gln Arg Ile Glu Gly Leu Gln Ile Ser Lys Ser
380 385 390
TTA AGT ATG TTT GGA CAG TCT ATA TCA GGA CAA ATT GAT GCA GAT AAT 1344
Leu Ser Met Phe Gly Gln Ser Ile Ser Gly Gln Ile Asp Ala Asp Asn
395 400 405

AAT GCC TAT GTA GAT GTA GCA GTT GGT GCT TTT CGG TCT GAT TCT GCT 1392
Asn Gly Tyr Val Asp Val Ala Val Gly Ala Phe Arg Ser Asp Ser Ala
410 415 420 425
GTC TTG CTA AGG ACA AGA CCT GTA GTA ATT GTT GAC GCT TCT TTA AGC 1440
Val Leu Leu Arg Thr Arg Pro Val Val Ile Val Asp Ala Ser Leu Ser
430 435 440
CAC CCT GAG TCA GTA AAT AGA ACG AAA TTT GAC TGT GTT GAA AAT GGA 1488
His Pro Glu Ser Val Asn Arg Thr Lys Phe Asp Cys Val Glu Asn Gly
445 450 455
TGG CCT TCT GTG TGC ATA GAT CTA ACA CTT TGT TTC TCA TAT AAG GGC 1536
Trp Pro Ser Val Cys Ile Asp Leu Thr Leu Cys Phe Ser Tyr Lys Gly
460 465 470
AAG GAA GTT CCA GGT TAC ATT GTT TTG TTT TAT AAC ATG AGT TTG GAT 1584
Lys Glu Val Pro Gly Tyr Ile Val Leu Phe Tyr Asn Met Ser Leu Asp
475 480 485
GTG AAC AGA AAG GCA GAG TCT CCA CCA AGA TTC TAT TTC TCT TCT AAT 1632
Val Asn Arg Lys Ala Glu Ser Pro Pro Arg Phe Tyr Phe Ser Ser Asn
490 495 500 505
GGA ACT TCT GAC GTG ATT ACA GGA AGC ATA CAG GTG TCC AGC AGA GAA 1680
Gly Thr Ser Asp Val Ile Thr Gly Ser Ile Gln Val Ser Ser Arg Glu
510 515 520
GCT AAC TGT AGA ACA CAT CAA GCA TTT ATG CGG AAA GAT GTG CGG GAC 1728
Ala Asn Cys Arg Thr His Gln Ala Phe Met Arg Lys Asp Val Arg Asp
525 530 535
ATC CTC ACC CCA ATT CAG ATT GAA GCT GCT TAC CAC CTT GGT CCT CAT 1776
Ile Leu Thr Pro Ile Gln Ile Glu Ala Ala Tyr His Leu Gly Pro His
540 545 550
GTC ATC AGT AAA CGA AGT ACA GAG GAA TTC CCA CCA CTT CAG CCA ATT 1824
Val Ile Ser Lys Arg Ser Thr Glu Glu Phe Pro Pro Leu Gln Pro Ile
555 560 565

CTT CAG CAG AAG AAA GAA AAA GAC ATA ATG AAA AAA ACA ATA AAC TTT 1872
Leu Gln Gln Lys Lys Glu Lys Asp Ile Met Lys Lys Thr Ile Asn Phe
570 575 580 585

GCA AGG TTT TGT GCC CAT GAA AAT TGT TCT GCT GAT TTA CAG GTT TCT 1920
Ala Arg Phe Cys Ala His Glu Asn Cys Ser Ala Asp Leu Gln Val Ser
590 595 600

GCA AAG ATT GGG TTT TTG AAG CCC CAT GAA AAT AAA ACA TAT CTT GCT 1968
Ala Lys Ile Gly Phe Leu Lys Pro His Glu Asn Lys Thr Tyr Leu Ala
605 610 615

GTT GGG AGT ATG AAG ACA TTG ATG TTG AAT GTG TCC TTG TTT AAT GCT 2016
Val Gly Ser Met Lys Thr Leu Met Leu Asn Val Ser Leu Phe Asn Ala
620 625 630

GGA GAT GAT GCA TAT GAA ACG ACT CTA CAT GTC AAA CTA CCC GTG GGT 2064
Gly Asp Asp Ala Tyr Glu Thr Thr Leu His Val Lys Leu Pro Val Gly
635 640 645

CTT TAT TTC ATT AAG ATT TTA GAG CTG GAA GAG AAG CAA ATA AAC TGT 2112
Leu Tyr Phe Ile Lys Ile Leu Glu Leu Glu Glu Lys Gln Ile Asn Cys
650 655 660 665

GAA GTC ACA GAT AAC TCT GGC GTG GTA CAA CTT GAC TGC AGT ATT GGC 2160
Glu Val Thr Asp Asn Ser Gly Val Val Gln Leu Asp Cys Ser Ile Gly
670 675 680

TAT ATA TAT GTA GAT CAT CTC TCA AGG ATA GAT ATT AGC TTT CTC CTG 2208
Tyr Ile Tyr Val Asp His Leu Ser Arg Ile Asp Ile Ser Phe Leu Leu
685 690 695

GAT GTG AGC TCA CTC AGC AGA GCG GAA GAG GAC CTC AGT ATC ACA GTG 2256
Asp Val Ser Ser Leu Ser Arg Ala Glu Glu Asp Leu Ser Ile Thr Val
700 705 710

CAT GCT ACC TGT GAA AAT GAA GAG GAA ATG GAC AAT CTA AAG CAC AGC 2304
His Ala Thr Cys Glu Asn Glu Glu Glu Met Asp Asn Leu Lys His Ser
715 720 725

```
AGA GTG ACT GTA GCA ATA CCT TTA AAA TAT GAG GTT AAG CTG ACT GTT    2352
Arg Val Thr Val Ala Ile Pro Leu Lys Tyr Glu Val Lys Leu Thr Val
730                 735                 740                 745
CAT GGG TTT GTA AAC CCA ACT TCA TTT GTG TAT GGA TCA AAT GAT GAA    2400
His Gly Phe Val Asn Pro Thr Ser Phe Val Tyr Gly Ser Asn Asp Glu
                750                 755                 760
AAT GAG CCT GAA ACG TGC ATG GTG GAG AAA ATG AAC TTA ACT TTC CAT    2448
Asn Glu Pro Glu Thr Cys Met Val Glu Lys Met Asn Leu Thr Phe His
            765                 770                 775
GTT ATC AAC ACT GGC AAT AGT ATG GCT CCC AAT GTT AGT GTG GAA ATA    2496
Val Ile Asn Thr Gly Asn Ser Met Ala Pro Asn Val Ser Val Glu Ile
        780                 785                 790
ATG GTA CCA AAT TCT TTT AGC CCC CAA ACT GAT AAG CTG TTC AAC ATT    2588
Met Val Pro Asn Ser Phe Ser Pro Gln Thr Asp Lys Leu Phe Asn Ile
    795                 800                 805
TTG GAT GTC CAG ACT ACT ACT GGA GAA TGC CAC TTT GAA AAT TAT CAA    2592
Leu Asp Val Gln Thr Thr Thr Gly Glu Cys His Phe Glu Asn Tyr Gln
810                 815                 820                 825
AGA GTG TGT GCA TTA GAG CAG CAA AAG AGT GCA ATG CAG ACC TTG AAA    2640
Arg Val Cys Ala Leu Glu Gln Gln Lys Ser Ala Met Gln Thr Leu Lys
                830                 835                 840
GGC ATA GTC CGG TTC TTG TCC AAG ACT GAT AAG AGG CTA TTG TAC TGC    2688
Gly Ile Val Arg Phe Leu Ser Lys Thr Asp Lys Arg Leu Leu Tyr Cys
            845                 850                 855
ATA AAA GCT GAT CCA CAT TGT TTA AAT TTC TTG TGT AAT TTT GGG AAA    2736
Ile Lys Ala Asp Pro His Cys Leu Asn Phe Leu Cys Asn Phe Gly Lys
        860                 865                 870
ATG GAA AGT GGA AAA GAA GCC AGT GTT CAT ATC CAA CTC GAA GGC CGG    2784
Met Glu Ser Gly Lys Glu Ala Ser Val His Ile Gln Leu Glu Gly Arg
    875                 880                 885
```

```
CCA TCC ATT TTA GAA ATG GAT GAG ACT TCA GCA CTC AAG TTT GAA ATA      2832
Pro Ser Ile Leu Glu Met Asp Glu Thr Ser Ala Leu Lys Phe Glu Ile
890                 895                 900                 905
AGA GCA ACA GGT TTT CCA GAG CCA AAT CCA AGA GTA ATT GAA CTA AAC      2880
Arg Ala Thr Gly Phe Pro Glu Pro Asn Pro Arg Val Ile Glu Leu Asn
                910                 915                 920
AAG GAT GAG AAT GTT GCG CAT GTT CTA CTG GAA GGA CTA CAT CAT CAA      2928
Lys Asp Glu Asn Val Ala His Val Leu Leu Glu Gly Leu His His Gln
            925                 930                 935
AGA CCC AAA CGT TAT TTC ACG GAT CCC GAG CTGCTGGAAG CAGGCTCAGC        2978
Arg Pro Lys Arg Tyr Phe Thr Asp Pro Glu
        940                 945
GCTCCTGCCT GGACGCATCC CGGCTATGCA GCCCCAGTCC AGGGCAGCAA GGCAGGCCCC  3038
GTCTGCCTCT TCACCCGGAG CCTCTGCCCG CCCCACTCAT GCTCAGGGAG AGGGTCTTCT  3098
GGCTTTTTCC CAGGCTCTGG GCAGGCACAG GCTAGGTGCC CCTAACCCAG GCCCTGCACA  3158
CAAAGGGGCA GGTGCTGGGC TCAGACCTGC CAAGAGCCAT ATCCGGGAGG ACCCTGCCCC  3218
TGACCTAAGC CCACCCCAAA GGCCAAACTC TCCACTCCCT CAGCTCGGAC ACCTTCTCTC  3278
CTCCCAGATT CCAGTAACTC CCAATCTTCT CTCTGCA GAG CCC AAA TCT TGT GAC   3333
                                         Glu Pro Lys Ser Cys Asp
                                                     950
AAA ACT CAC ACA TGC CCA CCG TGC CCA GGTAAGCCAG CCCAGGCCTC            3380
Lys Thr His Thr Cys Pro Pro Cys Pro
    955                 960
GCCCTCCAGC TCAAGGCGGG ACAGGTGCCC TAGAGTAGCC TGCATCCAGG GACAGGCCCC  3440
AGCCGGGTGC TGACACGTCC ACCTCCATCT CTTCCTCA GCA CCT GAA CTC CTG       3493
                                          Ala Pro Glu Leu Leu
                                                      965
GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC      3541
Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
        970                 975                 980
```

```
ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC    3589
Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
    985                 990                 995
CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG    3637
His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
1000                1005                1010                1015
GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG    3685
Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr
                1020                1025                1030
TAC CGG GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT    3733
Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
            1035                1040                1045
GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC    3781
Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro
        1050                1055                1060
ATC GAG AAA ACC ATC TCC AAA GCC AAA GGTGGGACCC GTGGGGTGCG          3828
Ile Glu Lys Thr Ile Ser Lys Ala Lys
    1065                1070
AGGGCCACAT GGACAGAGGC CGGCTCGGCC CACCCTCTGC CCTGAGAGTG ACCGCTGTAC  3888
CAACCTCTGT CCTACA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG      3937
                  Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu
                          1075                1080
CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC CAG GTC AGC CTG ACC TGC    3985
Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys
    1085                1090                1095
CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC    4033
Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser
1100                1105                1110                1115
```

AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAT 4081
Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp
1120 1125 1130
TCC GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC 4129
Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser
1135 1140 1145
AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT 4177
Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala
1150 1155 1160
CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA 4225
Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
1165 1170 1175
TGA 4228

Sequence No. 2
Length of sequence: 3463
Type of sequence: Nucleic acid
Sequence

ATG AAT TTA CAA CCA ATT TTC TGG ATT GGA CTG ATC AGT TCA GTT TGC 48
Met Asn Leu Gln Pro Ile Phe Trp Ile Gly Leu Ile Ser Ser Val Cys
-20 -15 -10 -5
TGT GTG TTT GCT CAA ACA GAT GAA AAT AGA TGT TTA AAA GCA AAT GCC 96
Cys Val Phe Ala Gln Thr Asp Glu Asn Arg Cys Leu Lys Ala Asn Ala
1 5 10
AAA TCA TGT GGA GAA TGT ATA CAA GCA GGG CCA AAT TGT GGG TGG TGC 144
Lys Ser Cys Gly Glu Cys Ile Gln Ala Gly Pro Asn Cys Gly Trp Cys
15 20 25
ACA AAT TCA ACA TTT TTA CAG GAA GGA ATG CCT ACT TCT GCA CGA TGT 192
Thr Asn Ser Thr Phe Leu Gln Glu Gly Met Pro Thr Ser Ala Arg Cys
30 35 40

```
GAT GAT TTA GAA GCC TTA AAA AAG AAG GGT TGC CCT CCA GAT GAC ATA     240
Asp Asp Leu Glu Ala Leu Lys Lys Lys Gly Cys Pro Pro Asp Asp Ile
 45                  50                  55                  60
GAA AAT CCC AGA GGC TCC AAA GAT ATA AAG AAA AAT AAA AAT GTA ACC     288
Glu Asn Pro Arg Gly Ser Lys Asp Ile Lys Lys Asn Lys Asn Val Thr
                 65                  70                  75
AAC CGT AGC AAA GGA ACA GCA GAG AAG CTC AAG CCA GAG GAT ATT CAT     336
Asn Arg Ser Lys Gly Thr Ala Glu Lys Leu Lys Pro Glu Asp Ile His
             80                  85                  90
CAG ATC CAA CCA CAG CAG TTG GTT TTG CGA TTA AGA TCA GGG GAG CCA     384
Gln Ile Gln Pro Gln Gln Leu Val Leu Arg Leu Arg Ser Gly Glu Pro
         95                 100                 105
CAG ACA TTT ACA TTA AAA TTC AAG AGA GCT GAA GAC TAT CCC ATT GAC     432
Gln Thr Phe Thr Leu Lys Phe Lys Arg Ala Glu Asp Tyr Pro Ile Asp
    110                 115                 120
CTC TAC TAC CTT ATG GAC CTG TCT TAT TCA ATG AAA GAC GAT TTG GAG     480
Leu Tyr Tyr Leu Met Asp Leu Ser Tyr Ser Met Lys Asp Asp Leu Glu
125                 130                 135                 140
AAT GTA AAA AGT CTT GGA ACA GAT CTG ATG AAT GAA ATG AGG AGG ATT     528
Asn Val Lys Ser Leu Gly Thr Asp Leu Met Asn Glu Met Arg Arg Ile
                145                 150                 155
ACT TCG GAC TTC AGA ATT GGA TTT GGC TCA TTT GTG GAA AAG ACT GTG     576
Thr Ser Asp Phe Arg Ile Gly Phe Gly Ser Phe Val Glu Lys Thr Val
            160                 165                 170
ATG CCT TAC ATT AGC ACA ACA CCA GCT AAG CTC AGG AAC CCT TGC ACA     624
Met Pro Tyr Ile Ser Thr Thr Pro Ala Lys Leu Arg Asn Pro Cys Thr
        175                 180                 185
AGT GAA CAG AAC TGC ACC ACC CCA TTT AGC TAC AAA AAT GTG CTC AGT     672
Ser Glu Gln Asn Cys Thr Thr Pro Phe Ser Tyr Lys Asn Val Leu Ser
    190                 195                 200
```

```
CTT ACT AAT AAA GGA GAA GTA TTT AAT GAA CTT GTT GGA AAA CAG CGC    720
Leu Thr Asn Lys Gly Glu Val Phe Asn Glu Leu Val Gly Lys Gln Arg
205                 210                 215                 220
ATA TCT GGA AAT TTG GAT TCT CCA GAA GGT GGT TTC GAT GCC ATC ATG    768
Ile Ser Gly Asn Leu Asp Ser Pro Glu Gly Gly Phe Asp Ala Ile Met
                225                 230                 235
CAA GTT GCA GTT TGT GGA TCA CTG ATT GGC TGG AGG AAT GTT ACA CGG    816
Gln Val Ala Val Cys Gly Ser Leu Ile Gly Trp Arg Asn Val Thr Arg
            240                 245                 250
CTG CTG GTG TTT TCC ACA GAT GCC GGG TTT CAC TTT GCT GGA GAT GGG    864
Leu Leu Val Phe Ser Thr Asp Ala Gly Phe His Phe Ala Gly Asp Gly
        255                 260                 265
AAA CTT GGT GGC ATT GTT TTA CCA AAT GAT GGA CAA TGT CAC CTG GAA    912
Lys Leu Gly Gly Ile Val Leu Pro Asn Asp Gly Gln Cys His Leu Glu
    270                 275                 280
AAT AAT ATG TAC ACA ATG AGC CAT TAT TAT GAT TAT CCT TCT ATT GCT    960
Asn Asn Met Tyr Thr Met Ser His Tyr Tyr Asp Tyr Pro Ser Ile Ala
285                 290                 295                 300
CAC CTT GTC CAG AAA CTG AGT GAA AAT AAT ATT CAG ACA ATT TTT GCA    1008
His Leu Val Gln Lys Leu Ser Glu Asn Asn Ile Gln Thr Ile Phe Ala
                305                 310                 315
GTT ACT GAA GAA TTT CAG CCT GTT TAC AAG GAG CTG AAA AAC TTG ATC    1056
Val Thr Glu Glu Phe Gln Pro Val Tyr Lys Glu Leu Lys Asn Leu Ile
            320                 325                 330
CCT AAG TCA GCA GTA GGA ACA TTA TCT GCA AAT TCT AGC AAT GTA ATT    1104
Pro Lys Ser Ala Val Gly Thr Leu Ser Ala Asn Ser Ser Asn Val Ile
        335                 340                 345
CAG TTG ATC ATT GAT GCA TAC AAT TCC CTT TCC TCA GAA GTC ATT TTG    1152
Gln Leu Ile Ile Asp Ala Tyr Asn Ser Leu Ser Ser Glu Val Ile Leu
    350                 355                 360
```

```
GAA AAC GGC AAA TTG TCA GAA GGA GTA ACA ATA AGT TAC AAA TCT TAC     1200
Glu Asn Gly Lys Leu Ser Glu Gly Val Thr Ile Ser Tyr Lys Ser Tyr
365                 370                 375                 380
TGC AAG AAC GGG GTG AAT GGA ACA GGG GAA AAT GGA AGA AAA TGT TCC     1248
Cys Lys Asn Gly Val Asn Gly Thr Gly Glu Asn Gly Arg Lys Cys Ser
                385                 390                 395
AAT ATT TCC ATT GGA GAT GAG GTT CAA TTT GAA ATT AGC ATA ACT TCA     1296
Asn Ile Ser Ile Gly Asp Glu Val Gln Phe Glu Ile Ser Ile Thr Ser
            400                 405                 410
AAT AAG TGT CCA AAA AAG GAT TCT GAC AGC TTT AAA ATT AGG CCT CTG     1344
Asn Lys Cys Pro Lys Lys Asp Ser Asp Ser Phe Lys Ile Arg Pro Leu
        415                 420                 425
GGC TTT ACG GAG GAA GTA GAG GTT ATT CTT CAG TAC ATC TGT GAA TGT     1392
Gly Phe Thr Glu Glu Val Glu Val Ile Leu Gln Tyr Ile Cys Glu Cys
    430                 435                 440
GAA TGC CAA AGC GAA GGC ATC CCT GAA AGT CCC AAG TGT CAT GAA GGA     1440
Glu Cys Gln Ser Glu Gly Ile Pro Glu Ser Pro Lys Cys His Glu Gly
445                 450                 455                 460
AAT GGG ACA TTT GAG TGT GGC GCG TGC AGG TGC AAT GAA GGG CGT GTT     1488
Asn Gly Thr Phe Glu Cys Gly Ala Cys Arg Cys Asn Glu Gly Arg Val
                465                 470                 475
GGT AGA CAT TGT GAA TGC AGC ACA GAT GAA GTT AAC AGT GAA GAC ATG     1536
Gly Arg His Cys Glu Cys Ser Thr Asp Glu Val Asn Ser Glu Asp Met
            480                 485                 490
GAT GCT TAC TGC AGG AAA GAA AAC AGT TCA GAA ATC TGC AGT AAC AAT     1584
Asp Ala Tyr Cys Arg Lys Glu Asn Ser Ser Glu Ile Cys Ser Asn Asn
        495                 500                 505
GGA GAG TGC GTC TGC GGA CAG TGT GTT TGT AGG AAG AGG GAT AAT ACA     1632
Gly Glu Cys Val Cys Gly Gln Cys Val Cys Arg Lys Arg Asp Asn Thr
    510                 515                 520
```

AAT GAA ATT TAT TCT GGC AAA TTC TGC GAG TGT GAT AAT TTC AAC TGT 1680
Asn Glu Ile Tyr Ser Gly Lys Phe Cys Glu Cys Asp Asn Phe Asn Cys
525 530 535 540
GAT AGA TCC AAT GGC TTA ATT TGT GGA GGA AAT GGT GTT TGC AAG TGT 1728
Asp Arg Ser Asn Gly Leu Ile Cys Gly Gly Asn Gly Val Cys Lys Cys
545 550 555
CGT GTG TGT GAG TGC AAC CCC AAC TAC ACT GGC AGT GCA TGT GAC TGT 1776
Arg Val Cys Glu Cys Asn Pro Asn Tyr Thr Gly Ser Ala Cys Asp Cys
560 565 570
TCT TTG GAT ACT AGT ACT TGT GAA GCC AGC AAC GGA CAG ATC TGC AAT 1824
Ser Leu Asp Thr Ser Thr Cys Glu Ala Ser Asn Gly Gln Ile Cys Asn
575 580 585
GGC CGG GGC ATC TGC GAG TGT GGT GTC TGT AAG TGT ACA GAT CCG AAG 1872
Gly Arg Gly Ile Cys Glu Cys Gly Val Cys Lys Cys Thr Asp Pro Lys
590 595 600
TTT CAA GGG CAA ACG TGT GAG ATG TGT CAG ACC TGC CTT GGT GTC TGT 1920
Phe Gln Gly Gln Thr Cys Glu Met Cys Gln Thr Cys Leu Gly Val Cys
605 610 615 620
GCT GAG CAT AAA GAA TGT GTT CAG TGC AGA GCC TTC AAT AAA GGA GAA 1968
Ala Glu His Lys Glu Cys Val Gln Cys Arg Ala Phe Asn Lys Gly Glu
625 630 635
AAG AAA GAC ACA TGC ACA CAG GAA TGT TCC TAT TTT AAC ATT ACC AAG 2016
Lys Lys Asp Thr Cys Thr Gln Glu Cys Ser Tyr Phe Asn Ile Thr Lys
640 645 650
GTA GAA AGT CGG GAC AAA TTA CCC CAG CCG GTC CAA CCT GAT CCT GTG 2064
Val Glu Ser Arg Asp Lys Leu Pro Gln Pro Val Gln Pro Asp Pro Val
655 660 665
TCC CAT TGT AAG GAG AAG GAT GTT GAC GAC TGT TGG TTC TAT TTT ACG 2112
Ser His Cys Lys Glu Lys Asp Val Asp Asp Cys Trp Phe Tyr Phe Thr
670 675 680

TAT TCA GTG AAT GGG AAC AAC GAG GTC ATG GTT CAT GTT GTG GAG AAT 2160
Tyr Ser Val Asn Gly Asn Asn Glu Val Met Val His Val Val Glu Asn
685 690 695 700
CCA GAG TGT CCC ACT GGT CCA GAG GAT CCC GAG CTGCTGGAAG CAGGCTCAGC 2213
Pro Glu Cys Pro Thr Gly Pro Glu Asp Pro Glu
705 710
GCTCCTGCCT GGACGCATCC CGGCTATGCA GCCCCAGTCC AGGGCAGCAA GGCAGGCCCC 2273
GTCTGCCTCT TCACCCGGAG CCTCTGCCCG CCCCACTCAT GCTCAGGGAG AGGGTCTTCT 2333
GGCTTTTTCC CAGGCTCTGG GCAGGCACAG GCTAGGTGCC CCTAACCCAG GCCCTGCACA 2393
CAAAGGGGCA GGTGCTGGGC TCAGACCTGC CAAGAGCCAT ATCCGGGAGG ACCCTGCCCC 2453
TGACCTAAGC CCACCCCAAA GGCCAAACTC TCCACTCCCT CAGCTCGGAC ACCTTCTCTC 2513
CTCCCAGATT CCAGTAACTC CCAATCTTCT CTCTGCA GAG CCC AAA TCT TGT GAC 2568
Glu Pro Lys Ser Cys Asp
715
AAA ACT CAC ACA TGC CCA CCG TGC CCA GGTAAGCCAG CCCAGGCCTC 2615
Lys Thr His Thr Cys Pro Pro Cys Pro
720 725
GCCCTCCAGC TCAAGGCGGG ACAGGTGCCC TAGAGTAGCC TGCATCCAGG GACAGGCCCC 2675
AGCCGGGTGC TGACACGTCC ACCTCCATCT CTTCCTCA GCA CCT GAA CTC CTG 2728
Ala Pro Glu Leu Leu
730
GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC 2776
Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
735 740 745
ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC 2824
Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
750 755 760
CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG 2872
His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
765 770 775

CTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG 2920
Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr
780 785 790 795

TAC CGG GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT 2968
Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
800 805 810

GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC 3016
Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro
815 820 825

ATC GAG AAA ACC ATC TCC AAA GCC AAA GGTGGGACCC GTGGGGTGCG 3063
Ile Glu Lys Thr Ile Ser Lys Ala Lys
830 835

AGGGCCACAT GGACAGAGGC CGGCTCGGCC CACCCTCTGC CCTGAGAGTG ACCGCTGTAC 3123

CAACCTCTGT CCTACA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG 3172
Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu
840 845

CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC CAG GTC AGC CTG ACC TGC 3220
Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys
850 855 860

CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC 3268
Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser
865 870 875

AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAT 3316
Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp
880 885 890 895

TCC GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC 3364
Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser
900 905 910

AGG TCG CAC CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT 3412
Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala
915 920 925

CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA 3460
Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
930 935 940

TGA 3463

Sequence No. 3

Length of sequence: 13

Type of sequence: Amino acid

Topology: Linear

Kind of sequence: Peptide

Sequence

Cys Leu His Gly Pro Glu Ile Leu Asp Val Pro Ser Thr
1 5 10

Sequence No. 4

Length of sequence: 31

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

GCGGATCCCG AGCTGCTGGA AGCAGGCTCA G 31

Sequence No. 5

Length of sequence: 27

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

CCTCTAGACG GCCGTCGCAC TCATTTA 27

Sequence No. 6

Length of sequence: 73

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

CTAGACCACC ATGTTCCCCA CCGAGAGCGC ATGGCTTGGG AAGCGAGGCG CGAACCCGGG

CCCCGGAGCT GCA 73

Sequence No. 7

Length of sequence: 65

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

GCTTCGGGGC CCGGGTTCGC GCCTCGCTTC CCAAGCCATG CGCTCTCGGT GGGGAACATG

GTGGT 65

Sequence No. 8

Length of sequence: 51

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

CTCCGGGAGA CGGTGATGCT GTTGCTGTGC CTGGGGGTCC CGACCGGCAG G 51

Sequence No. 9

Length of sequence: 55

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Straight chain

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

CCTGCCGGTC GGGACCCCCA GGCACAGCAA CAGCATCACC GTCTCCCGGA GTCGA 55

Sequence No. 10

Length of sequence: 37

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

CACTGCAGGC AGGCCTTACA ACGTGGACAC TGAGAGC 37

Sequence No. 11

Length of sequence: 22

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

GCAGAAACCT GTAAATCAGC AG 22

Sequence No. 12

Length of sequence: 22

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

GCATTTATGC GGAAAGATGT GC 22

Sequence No. 13

Length of sequence: 29

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

CGGGATCCGT GAAATAACGT TTGGGTCTT 29

Sequence No. 14

Length of sequence: 22

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

GCGGAAAAGA TGAATTTACA AC 22

Sequence No. 15

Length of sequence: 27

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

GTGGCATCCT CTGGACCAGT GGCACAC 27

Sequence No. 16

Length of sequence: 10

Type of sequence: Amino acid

Topology: Linear

Kind of sequence: Peptide

Sequence

Gly Pro Glu Ilè Leu Asp Val Pro Ser Thr
1 5 10

Sequence No. 17

Length of sequence: 10

Type of sequence: Amino acid

Topology: Linear

Kind of sequence: Peptide

Sequence

Gly Pro Glu Ile Leu Glu Val Pro Ser Thr
1 5 10

Sequence No. 18

Length of sequence: 6

Type of sequence: Amino acid

Topology: Linear

Kind of sequence: Peptide

Sequence

Gly Arg Gly Asp Ser Pro
1 5

Sequence No. 19

Length of sequence: 4675

Type of sequence: Nucleic acid

Sequence

```
ATG GGG CCA GAA CGG ACA GGG GCC GCG CCG CTG CCG CTG CTG CTG GTG    48
Met Gly Pro Glu Arg Thr Gly Ala Ala Pro Leu Pro Leu Leu Leu Val
                -25                 -20                 -15
TTA GCG CTC AGT CAA GGC ATT TTA AAT TGT TGT TTG GCC TAC AAT GTT    96
Leu Ala Leu Ser Gln Gly Ile Leu Asn Cys Cys Leu Ala Tyr Asn Val
            -10                 -5                  1
GGT CTC CCA GAA GCA AAA ATA TTT TCC GGT CCT TCA AGT GAA CAG TTT   144
Gly Leu Pro Glu Ala Lys Ile Phe Ser Gly Pro Ser Ser Glu Gln Phe
    5                   10                  15
GGG TAT GCA GTG CAG CAG TTT ATA AAT CCA AAA GGC AAC TGG TTA CTG   192
Gly Tyr Ala Val Gln Gln Phe Ile Asn Pro Lys Gly Asn Trp Leu Leu
20                  25                  30                  35
GTT GGT TCA CCC TGG AGT GGC TTT CCT GAG AAC CGA ATG GGA GAT GTG   240
Val Gly Ser Pro Trp Ser Gly Phe Pro Glu Asn Arg Met Gly Asp Val
                40                  45                  50
TAT AAA TGT CCT GTT GAC CTA TCC ACT GCC ACA TGT GAA AAA CTA AAT   288
Tyr Lys Cys Pro Val Asp Leu Ser Thr Ala Thr Cys Glu Lys Leu Asn
            55                  60                  65
TTG CAA ACT TCA ACA AGC ATT CCA AAT GTT ACT GAG ATG AAA ACC AAC   336
Leu Gln Thr Ser Thr Ser Ile Pro Asn Val Thr Glu Met Lys Thr Asn
        70                  75                  80
```

```
ATG AGC CTC GGC TTG ATC CTC ACC AGG AAC ATG GGA ACT GGA GGT TTT      384
Met Ser Leu Gly Leu Ile Leu Thr Arg Asn Met Gly Thr Gly Gly Phe
     85                  90                  95
CTC ACA TGT GGT CCT CTG TGG GCA CAG CAA TGT GGG AAT CAG TAT TAC      432
Leu Thr Cys Gly Pro Leu Trp Ala Gln Gln Cys Gly Asn Gln Tyr Tyr
100                 105                 110                 115
ACA ACG GGT GTG TGT TCT GAC ATC AGT CCT GAT TTT CAG CTC TCA GCC      480
Thr Thr Gly Val Cys Ser Asp Ile Ser Pro Asp Phe Gln Leu Ser Ala
                120                 125                 130
AGC TTC TCA CCT GCA ACT CAG CCC TGC CCT TCC CTC ATA GAT GTT GTG      528
Ser Phe Ser Pro Ala Thr Gln Pro Cys Pro Ser Leu Ile Asp Val Val
            135                 140                 145
GTT GTG TGT GAT GAA TCA AAT AGT ATT TAT CCT TGG GAT GCA GTA AAG      576
Val Val Cys Asp Glu Ser Asn Ser Ile Tyr Pro Trp Asp Ala Val Lys
        150                 155                 160
AAT TTT TTG GAA AAA TTT GTA CAA GGC CTT GAT ATA GGC CCC ACA AAG      624
Asn Phe Leu Glu Lys Phe Val Gln Gly Leu Asp Ile Gly Pro Thr Lys
    165                 170                 175
ACA CAG GTG GGG TTA ATT CAG TAT GCC AAT AAT CCA AGA GTT GTG TTT      672
Thr Gln Val Gly Leu Ile Gln Tyr Ala Asn Asn Pro Arg Val Val Phe
180                 185                 190                 195
AAC TTG AAC ACA TAT AAA ACC AAA GAA GAA ATG ATT GTA GCA ACA TCC      720
Asn Leu Asn Thr Tyr Lys Thr Lys Glu Glu Met Ile Val Ala Thr Ser
                200                 205                 210
CAG ACA TCC CAA TAT GGT GGG GAC CTC ACA AAC ACA TTC GGA GCA ATT      768
Gln Thr Ser Gln Tyr Gly Gly Asp Leu Thr Asn Thr Phe Gly Ala Ile
            215                 220                 225
CAA TAT GCA AGA AAA TAT GCC TAT TCA GCA GCT TCT GGT GGG CGA CGA      816
Gln Tyr Ala Arg Lys Tyr Ala Tyr Ser Ala Ala Ser Gly Gly Arg Arg
        230                 235                 240
```

AGT GCT ACG AAA GTA ATG GTA GTT GTA ACT GAC GGT GAA TCA CAT GAT 864
Ser Ala Thr Lys Val Met Val Val Val Thr Asp Gly Glu Ser His Asp
245 250 255

GGT TCA ATG TTG AAA GCT GTG ATT GAT CAA TGC AAC CAT GAC AAT ATA 912
Gly Ser Met Leu Lys Ala Val Ile Asp Gln Cys Asn His Asp Asn Ile
260 265 270 275

CTG AGG TTT GGC ATA GCA GTT CTT GGG TAC TTA AAC AGA AAC GCC CTT 960
Leu Arg Phe Gly Ile Ala Val Leu Gly Tyr Leu Asn Arg Asn Ala Leu
280 285 290

GAT ACT AAA AAT TTA ATA AAA GAA ATA AAA GCG ATC GCT AGT ATT CCA 1008
Asp Thr Lys Asn Leu Ile Lys Glu Ile Lys Ala Ile Ala Ser Ile Pro
295 300 305

ACA GAA AGA TAC TTT TTC AAT GTG TCT GAT GAA GCA GCT CTA CTA GAA 1056
Thr Glu Arg Tyr Phe Phe Asn Val Ser Asp Glu Ala Ala Leu Leu Glu
310 315 320

AAG GCT GGG ACA TTA GGA GAA CAA ATT TTC AGC ATT GAA GGT ACT GTT 1104
Lys Ala Gly Thr Leu Gly Glu Gln Ile Phe Ser Ile Glu Gly Thr Val
325 330 335

CAA GGA GGA GAC AAC TTT CAG ATG GAA ATG TCA CAA GTG GGA TTC AGT 1152
Gln Gly Gly Asp Asn Phe Gln Met Glu Met Ser Gln Val Gly Phe Ser
340 345 350 355

GCA GAT TAC TCT TCT CAA AAT GAT ATT CTG ATG CTG GGT GCA GTG GGA 1200
Ala Asp Tyr Ser Ser Gln Asn Asp Ile Leu Met Leu Gly Ala Val Gly
360 365 370

GCT TTT GGC TGG AGT GGG ACC ATT GTC CAG AAG ACA TCT CAT GGC CAT 1248
Ala Phe Gly Trp Ser Gly Thr Ile Val Gln Lys Thr Ser His Gly His
375 380 385

TTG ATC TTT CCT AAA CAA GCC TTT GAC CAA ATT CTG CAG GAC AGA AAT 1296
Leu Ile Phe Pro Lys Gln Ala Phe Asp Gln Ile Leu Gln Asp Arg Asn
390 395 400

CAC ACT TCA TAT TTA GGT TAC TCT GTG GCT GCA ATT TCT ACT GGA GAA 1344
His Ser Ser Tyr Leu Gly Tyr Ser Val Ala Ala Ile Ser Thr Gly Glu
405 410 415

AGC ACT CAC TTT GTT GCT GGT GCT CCT CGG GCA AAT TAT ACC GGC CAG 1392
Ser Thr His Phe Val Ala Gly Ala Pro Arg Ala Asn Tyr Thr Gly Gln
420 425 430 435

ATA GTG CTA TAT AGT GTG AAT GAG AAT GGC AAT ATC ACG GTT ATT CAG 1440
Ile Val Leu Tyr Ser Val Asn Glu Asn Gly Asn Ile Thr Val Ile Gln
440 445 450

GCT CAC CGA GGT GAC CAG ATT GGC TCC TAT TTT GGT AGT GTG CTG TGT 1488
Ala His Arg Gly Asp Gln Ile Gly Ser Tyr Phe Gly Ser Val Leu Cys
455 460 465

TCA GTT GAT GTG GAT AAA GAC ACC ATT ACA GAC GTG CTC TTG GTA GGT 1536
Ser Val Asp Val Asp Lys Asp Thr Ile Thr Asp Val Leu Leu Val Gly
470 475 480

GCA CCA ATG TAC ATG AGT GAC CTA AAG AAA GAG GAA GGA AGA GTC TAC 1584
Ala Pro Met Tyr Met Ser Asp Leu Lys Lys Glu Glu Gly Arg Val Tyr
485 490 495

CTG TTT ACT ATC AAA AAG GGC ATT TTG GGT CAG CAC CAA TTT CTT GAA 1632
Leu Phe Thr Ile Lys Lys Gly Ile Leu Gly Gln His Gln Phe Leu Glu
500 505 510 515

GGC CCC GAG GGC ATT GAA AAC ACT CGA TTT GGT TCA GCA ATT GCA GCT 1680
Gly Pro Glu Gly Ile Glu Asn Thr Arg Phe Gly Ser Ala Ile Ala Ala
520 525 530

CTT TCA GAC ATC AAC ATG GAT GGC TTT AAT GAT GTG ATT GTT GGT TCA 1728
Leu Ser Asp Ile Asn Met Asp Gly Phe Asn Asp Val Ile Val Gly Ser
535 540 545

CCA CTA GAA AAT CAG AAT TCT GGA GCT GTA TAC ATT TAC AAT GGT CAT 1776
Pro Leu Glu Asn Gln Asn Ser Gly Ala Val Tyr Ile Tyr Asn Gly His
550 555 560

CAG GGC ACT ATC CGC ACA AAG TAT TCC CAG AAA ATC TTG GGA TCC GAT 1824
Gln Gly Thr Ile Arg Thr Lys Tyr Ser Gln Lys Ile Leu Gly Ser Asp
565 570 575

GGA GCC TTT AGG AGC CAT CTC CAG TAC TTT GGG AGG TCC TTG GAT GGC 1872
Gly Ala Phe Arg Ser His Leu Gln Tyr Phe Gly Arg Ser Leu Asp Gly
580 585 590 595

TAT GGA GAT TTA AAT GGC GAT TCC ATC ACC GAT GTG TCT ATT GGT GCC 1920
Tyr Gly Asp Leu Asn Gly Asp Ser Ile Thr Asp Val Ser Ile Gly Ala
600 605 610

TTT GGA CAA GTG GTT CAA CTC TGG TCA CAA AGT ATT GCT GAT GTA GCT 1968
Phe Gly Gln Val Val Gln Leu Trp Ser Gln Ser Ile Ala Asp Val Ala
615 620 625

ATA GAA GCT TCA TTC ACA CCA GAA AAA ATC ACT TTG GTC AAC AAG AAT 2016
Ile Glu Ala Ser Phe Thr Pro Glu Lys Ile Thr Leu Val Asn Lys Asn
630 635 640

GCT CAG ATA ATT CTC AAA CTC TGC TTC AGT GCA AAG TTC AGA CCT ACT 2064
Ala Gln Ile Ile Leu Lys Leu Cys Phe Ser Ala Lys Phe Arg Pro Thr
645 650 655

AAG CAA AAC AAT CAA GTG GCC ATT GTA TAT AAC ATC ACA CTT GAT GCA 2112
Lys Gln Asn Asn Gln Val Ala Ile Val Tyr Asn Ile Thr Leu Asp Ala
660 665 670 675

GAT GGA TTT TCA TCC AGA GTA ACC TCC AGG GGG TTA TTT AAA GAA AAC 2160
Asp Gly Phe Ser Ser Arg Val Thr Ser Arg Gly Leu Phe Lys Glu Asn
680 685 690

AAT GAA AGG TGC CTG CAG AAG AAT ATG GTA GTA AAT CAA GCA CAG AGT 2208
Asn Glu Arg Cys Leu Gln Lys Asn Met Val Val Asn Gln Ala Gln Ser
695 700 705

TGC CCC GAG CAC ATC ATT TAT ATA CAG GAG CCC TCT GAT GTT GTC AAC 2256
Cys Pro Glu His Ile Ile Tyr Ile Gln Glu Pro Ser Asp Val Val Asn
710 715 720

TCT TTG GAT TTG CGT GTG GAC ATC AGT CTG GAA AAC CCT GGC ACT AGC 2304
Ser Leu Asp Leu Arg Val Asp Ile Ser Leu Glu Asn Pro Gly Thr Ser
725 730 735

CCT GCC CTT GAA GCC TAT TCT GAG ACT GCC AAG GTC TTC AGT ATT CCT 2352
Pro Ala Leu Glu Ala Tyr Ser Glu Thr Ala Lys Val Phe Ser Ile Pro
740 745 750 755

TTC CAC AAA GAC TGT GGT GAG GAT GGA CTT TGC ATT TCT GAT CTA GTC 2400
Phe His Lys Asp Cys Gly Glu Asp Gly Leu Cys Ile Ser Asp Leu Val
760 765 770

CTA GAT GTC CGA CAA ATA CCA GCT GCT CAA GAA CAA CCC TTT ATT GTC 2448
Leu Asp Val Arg Gln Ile Pro Ala Ala Gln Glu Gln Pro Phe Ile Val
775 780 785

AGC AAC CAA AAC AAA AGG TTA ACA TTT TCA GTA ACA CTG AAA AAT AAA 2496
Ser Asn Gln Asn Lys Arg Leu Thr Phe Ser Val Thr Leu Lys Asn Lys
790 795 800

AGG GAA AGT GCA TAC AAC ACT GGA ATT GTT GTT GAT TTT TCA GAA AAC 2544
Arg Glu Ser Ala Tyr Asn Thr Gly Ile Val Val Asp Phe Ser Glu Asn
805 810 815

TTG TTT TTT GCA TCA TTC TCC CTA CCG GTT GAT GGG ACA GAA GTA ACA 2592
Leu Phe Phe Ala Ser Phe Ser Leu Pro Val Asp Gly Thr Glu Val Thr
820 825 830 835

TGC CAG GTG GCT GCA TCT CAG AAG TCT GTT GCC TGC GAT GTA GGC TAC 2640
Cys Gln Val Ala Ala Ser Gln Lys Ser Val Ala Cys Asp Val Gly Tyr
840 845 850

CCT GCT TTA AAG AGA GAA CAA CAG GTG ACT TTT ACT ATT AAC TTT GAC 2688
Pro Ala Leu Lys Arg Glu Gln Gln Val Thr Phe Thr Ile Asn Phe Asp
855 860 865

TTC AAT CTT CAA AAC CTT CAG AAT CAG GCG TCT CTC AGT TTC CAA GCC 2736
Phe Asn Leu Gln Asn Leu Gln Asn Gln Ala Ser Leu Ser Phe Gln Ala
870 875 880

```
TTA AGT GAA AGC CAA GAA GAA AAC AAG GCT GAT AAT TTG GTC AAC CTC    2784
Leu Ser Glu Ser Gln Glu Glu Asn Lys Ala Asp Asn Leu Val Asn Leu
    885                 890                 895
AAA ATT CCT CTC CTG TAT GAT GCT GAA ATT CAC TTA ACA AGA TCT ACC    2832
Lys Ile Pro Leu Leu Tyr Asp Ala Glu Ile His Leu Thr Arg Ser Thr
900                 905                 910                 915
AAC ATA AAT TTT TAT GAA ATC TCT TCG GAT GGG AAT GTT CCT TCA ATC    2880
Asn Ile Asn Phe Tyr Glu Ile Ser Ser Asp Gly Asn Val Pro Ser Ile
                920                 925                 930
GTG CAC AGT TTT GAA GAT GTT GGT CCA AAA TTC ATC TTC TCC CTG AAG    2928
Val His Ser Phe Glu Asp Val Gly Pro Lys Phe Ile Phe Ser Leu Lys
            935                 940                 945
GTA ACA ACA GGA AGT GTT CCA GTA AGC ATG GCA ACT GTA ATC ATC CAC    2976
Val Thr Thr Gly Ser Val Pro Val Ser Met Ala Thr Val Ile Ile His
        950                 955                 960
ATC CCT CAG TAT ACC AAA GAA AAG AAC CCA CTG ATG TAC CTA ACT GGG    3024
Ile Pro Gln Tyr Thr Lys Glu Lys Asn Pro Leu Met Tyr Leu Thr Gly
    965                 970                 975
GTG CAA ACA GAC AAG GCT GGT GAC ATC AGT TGT AAT GCA GAT ATC AAT    3072
Val Gln Thr Asp Lys Ala Gly Asp Ile Ser Cys Asn Ala Asp Ile Asn
980                 985                 990                 995
CCA CTG AAA ATA GGA CAA ACA TCT TCT TCT GTA TCT TTC AAA AGT GAA    3120
Pro Leu Lys Ile Gly Gln Thr Ser Ser Ser Val Ser Phe Lys Ser Glu
                1000                1005                1010
AAT TTC AGG CAC ACC AAA GAA TTG AAC TGC AGA ACT GCT TCC TGT AGT    3168
Asn Phe Arg His Thr Lys Glu Leu Asn Cys Arg Thr Ala Ser Cys Ser
            1015                1020                1025
AAT GTT ACC TGC TGG TTG AAA GAC GTT CAC ATG AAA GGA GAA TAC TTT    3216
Asn Val Thr Cys Trp Leu Lys Asp Val His Met Lys Gly Glu Tyr Phe
        1030                1035                1040
```

GTT AAT GTG ACT ACC AGA ATT TGG AAC GGG ACT TTC GCA TCA TCA ACG 3264
Val Asn Val Thr Thr Arg Ile Trp Asn Gly Thr Phe Ala Ser Ser Thr
1045 1050 1055

TTC CAG ACA GTA CAG CTA ACG GCA GCT GCA GAA ATC AAC ACC TAT AAC 3312
Phe Gln Thr Val Gln Leu Thr Ala Ala Ala Glu Ile Asn Thr Tyr Asn
1060 1065 1070 1075

CCT GAG ATA TAT GTG ATT GAA GAT AAC ACT GTT ACG ATT CCC CTG ATG 3360
Pro Glu Ile Tyr Val Ile Glu Asp Asn Thr Val Thr Ile Pro Leu Met
1080 1085 1090

ATA ATG AAA CCT GAT GAG AAA GCC GAA GTA CCA ACA GAT CCC GAG 3405
Ile Met Lys Pro Asp Glu Lys Ala Glu Val Pro Thr Asp Pro Glu
1095 1100 1105

CTGCTGGAAG CAGGCTCAGC GCTCCTGCCT GGACGCATCC CGGCTATGCA GCCCCAGTCC 3465
AGGGCAGCAA GGCAGGCCCC GTCTGCCTCT TCACCCGGAG CCTCTGCCCG CCCCACTCAT 3525
GCTCAGGGAG AGGGTCTTCT GGCTTTTTCC CAGGCTCTGG GCAGGCACAG GCTAGGTGCC 3585
CCTAACCCAG GCCCTGCACA CAAAGGGGCA GGTGCTGGGC TCAGACCTGC CAAGAGCCAT 3645
ATCCGGGAGG ACCCTGCCCC TGACCTAAGC CCACCCCAAA GGCCAAACTC TCCACTCCCT 3705
CAGCTCGGAC ACCTTCTCTC CTCCCAGATT CCAGTAACTC CCAATCTTCT CTCTGCA 3762

GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA 3807
Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro
1110 1115 1120

GGTAAGCCAG CCCAGGCCTC GCCCTCCAGC TCAAGGCGGG ACAGGTGCCC TAGAGTAGCC 3867
TGCATCCAGG GACAGGCCCC AGCCGGGTGC TGACACGTCC ACCTCCATCT CTTCCTCA 3925

GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA AAA 3973
Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys
1125 1130 1135

CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG 4021
Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val
1140 1145 1150

GTG GTG GAC GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC 4069
Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr
1155 1160 1165

GTG GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG 4117
Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu
1170 1175 1180 1185

CAG TAC AAC AGC ACG TAC CGG GTG GTC AGC GTC CTC ACC GTC CTG CAC 4165
Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His
1190 1195 1200

CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA 4213
Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys
1205 1210 1215

GCC CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA 4255
Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys
1220 1225 1230

GGTGGGACCC GTGGGGTGCG AGGGCCACAT GGACAGAGGC CGGCTCGGCC CACCCTCTGC 4315
CCTGAGAGTG ACCGCTGTAC CAACCTCTGT CCTACA GGG CAG CCC CGA GAA CCA 4369
Gly Gln Pro Arg Glu Pro
1235

CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC CAG 4417
Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln
1240 1245 1250

GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC GCC 4465
Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala
1255 1260 1265

GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC ACG 4513
Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr
1270 1275 1280 1285

CCT CCC GTG CTG GAT TCC GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC 4561
Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu
1290 1295 1300
ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC 4609
Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser
1305 1310 1315
GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC 4657
Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser
1320 1325 1330
CTG TCT CCG GGT AAA TGA 4675
Leu Ser Pro Gly Lys
1335

Sequence No. 20
Length of sequence: 27
Type of sequence: Nucleic acid
Number of strands: Single
Topology: Linear
Kind of sequence: Other nucleic acid, synthetic DNA
Sequence
GCTCGAGCAA ACCCAGCGCA ACTACGG 27

Sequence No. 21
Length of sequence: 21
Type of sequence: Nucleic acid
Number of strands: Single
Topology: Linear
Kind of sequence: Other nucleic acid, synthetic DNA
Sequence
ATAGTGCCCT GATGACCATT G 21

Sequence No. 22

Length of sequence: 22

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

GATGGCTTTA ATGATGTGAT TG 22

Sequence No. 23

Length of sequence: 21

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

TGTTGGTACT TCGGCTTTCT C 21

Sequence No. 24

Length of sequence: 8

Type of sequence: Amino acid

Topology: Circular

Kind of sequence: Peptide

Sequence

Cys Ile Pro Glu Leu Ile Val Cys
1 5

Sequence No. 25

Length of sequence: 8

Type of sequence: Amino acid

Topology: Circular

Kind of sequence: Peptide

Sequence

Cys Met Arg Tyr Thr Ser Ala Cys

1 5

Sequence No. 26

Length of sequence: 8

Type of sequence: Amino acid

Topology: Circular

Kind of sequence: Peptide

Sequence

Cys Glu Trp Met Lys Arg Phe Cys

1 5

Sequence No. 27

Length of sequence: 8

Type of sequence: Amino acid

Topology: Circular

Kind of sequence: Peptide

Sequence

Cys Tyr Thr Thr Arg Leu Lys Cys

1 5

Sequence No. 28

Length of sequence: 8

Type of sequence: Amino acid

Topology: Circular

Kind of sequence: Peptide

Sequence

Cys Leu Arg Tyr Ser Val Pro Cys
1 5

Sequence No. 29
Length of sequence: 8
Type of sequence: Amino acid
Topology: Circular
Kind of sequence: Peptide
Sequence

Cys Ile Val Asn Arg Leu Gly Cys
1 5

Sequence No. 30
Length of sequence: 8
Type of sequence: Amino acid
Topology: Circular
Kind of sequence: Peptide
Sequence

Cys Gly Leu Gln Ala Leu Pro Cys
1 5

Sequence No. 31
Length of sequence: 8
Type of sequence: Amino acid
Topology: Circular
Kind of sequence: Peptide
Sequence

Cys Lys Leu Lys Gly Thr Met Cys
1 5

Claims

1. A chimeric protein comprising the α chain or β chain of an integrin and the heavy chain or light chain of an immunoglobulin.

2. A chimeric protein heterodimer complex, characterized in that a chimeric protein stated in claim 1 comprising the α chain of an integrin and the heavy chain or light chain of an immunoglobulin and a chimeric protein stated in claim 1 comprising the β chain of the integrin and the heavy chain or light chain of the immunoglobulin are associated with each other.

3. A chimeric protein heterodimer complex, according to claim 2, wherein the chimeric proteins stated in claim 1 are associated with each other in any of the following combinations (1), (2) and (3):

   (1) An α chain • immunoglobulin heavy chain-β chain • immunoglobulin heavy chain chimeric protein heterodimer complex, in which a chimeric protein comprising the α chain of an integrin and the heavy chain of an immunoglobulin and a chimeric protein comprising the β chain of the integrin and the heavy chain of the immunoglobulin are associated with each other.
   (2) An α chain • immunoglobulin heavy chain-β chain • immunoglobulin light chain chimeric protein heterodimer complex, in which a chimeric protein comprising the α chain of an integrin and the heavy chain of an immunoglobulin and a chimeric protein comprising the β chain of the integrin and the light chain of the immunoglobulin are associated with each other.
   (3) An α chain • immunoglobulin light chain-β chain • immunoglobulin heavy chain chimeric protein heterodimer complex, in which a chimeric protein comprising the α chain of an integrin and the light chain of an immunoglobulin and a chimeric protein comprising of the β chain of the integrin and the heavy chain of the immunoglobulin are associated with each other.

4. A chimeric protein or a chimeric protein heterodimer complex, according to any one of claims 1 through 3, wherein the α chain of an integrin is $\alpha1$, $\alpha2$, $\alpha3$, $\alpha4$, $\alpha5$, $\alpha6$, $\alpha7$, $\alpha8$, $\alpha9$, αv, αL, αM, αX, αIIb or αE.

5. A chimeric protein or a chimeric protein heterodimer complex, according to any one of claims 1 through 3, wherein the β chain of an integrin is $\beta1$, $\beta2$, $\beta3$, $\beta4$, $\beta5$, $\beta6$, $\beta7$ or $\beta8$.

6. A chimeric protein heterodimer complex, according to claim 2 or 3, wherein the α chain of an integrin is $\alpha4$ or $\alpha2$ and the β chain is $\beta1$.

7. A chimeric protein or a chimeric protein heterodimer complex, according to any one of claims 1 through 3, wherein the chimeric protein comprising the $\alpha4$ of an integrin and the heavy chain of an immunoglobulin is identified as the amino acid sequence of sequence No. 1.

8. A chimeric protein or a chimeric protein heterodimer complex, according to any one of claims 1 through 3, wherein the chimeric protein comprising the $\alpha2$ of an integrin and the heavy chain of an immunoglobulin is identified as the amino acid sequence of sequence No. 19.

9. A chimeric protein or a chimeric protein heterodimer complex, according to any one of claims 1 through 3, wherein the chimeric protein comprising the $\beta1$ of an integrin and the heavy chain of an immunoglobulin is identified as the amino acid sequence of sequence No. 2.

10. A DNA coding for a chimeric protein stated in claim 1.

11. A DNA coding for a chimeric protein stated in claim 1, wherein the α chain of an integrin is $\alpha1$, $\alpha2$, $\alpha3$, $\alpha4$, $\alpha5$, $\alpha6$, $\alpha7$, $\alpha8$, $\alpha9$, αv, αL, αM, αX, αIIb or αE.

12. A DNA coding for a chimeric protein stated in claim 1, wherein the β chain of an integrin is $\beta1$, $\beta2$, $\beta3$, $\beta4$, $\beta5$, $\beta6$, $\beta7$ or $\beta8$.

13. A DNA, according to claim11, which is identified as the nucleotide sequence of sequence No. 1 or 19.

14. A DNA, according to claim 12, which is identified as the nucleotide sequence of sequence No. 2.

15. A recombinant vector, wherein a DNA stated in claim 10 is functionally linked to an expression control sequence.

16. A recombinant vector, wherein a DNA stated in claim 11 is functionally linked to an expression control sequence.

17. A recombinant vector, wherein a DNA stated in claim 12 is functionally linked to an expression control sequence.

18. A recombinant vector, wherein a DNA stated in claim 13 is functionally linked to an expression control sequence.

19. A recombinant vector, wherein the DNA stated in claim 14 is functionally linked to an expression control sequence.

20. An animal cell, comprising being transfected simultaneously by a recombinant vector in which a DNA coding for a chimeric protein comprising the α chain of an integrin and the heavy chain or light chain of an immunoglobulin is functionally linked to an expression control sequence, and a recombinant vector in which a DNA coding for a chimeric protein comprising the β chain of the integrin and the heavy chain or light chain of the immunoglobulin is functionally linked to an expression control sequence.

21. An animal cell, according to claim 20, which is transfected simultaneously by the recombinant vectors stated in claims 16 and 17.

22. An animal cell, according to claim 20, which is transfected simultaneously by the recombinant vectors stated in claims 18 and 19.

23. A method for producing the chimeric protein heterodimer complex stated in claim 2, comprising culturing the animal cell stated in claim 20.

24. A drug, comprising a chimeric protein or chimeric protein heterodimer complex stated in any one of claims 1 through 9.

25. A drug composition, comprising a chimeric protein or chimeric protein heterodimer complex stated in any one of claims 1 through 9.

26. A platelet substitute, comprising an isolated extracellular matrix receptor as an active ingredient.

27. A platelet substitute, according to claim 26, wherein the extracellular matrix receptor is an integrin.

28. A platelet substitute, according to claim 27, wherein the α chain of an integrin is $\alpha1$, $\alpha2$, $\alpha3$, $\alpha4$, $\alpha5$, $\alpha6$, $\alpha7$, $\alpha8$, $\alpha9$, αv, αL, αM, αX, αIIb or αE.

29. A platelet substitute, according to claim 27, wherein the β chain of an integrin is $\beta1$, $\beta2$, $\beta3$, $\beta4$, $\beta5$, $\beta6$, $\beta7$ or $\beta8$.

30. A platelet substitute, according to claim 27, wherein the integrin is integrin $\alpha2\beta1$.

31. A platelet substitute, according to claim 26, wherein the extracellular matrix receptor is a chimeric protein heterodimer complex comprising an extracellular matrix receptor and an immunoglobulin.

32. A platelet substitute, according to claim 31, wherein the chimeric protein heterodimer complex is a chimeric protein heterodimer complex comprising an integrin and an immunoglobulin.

33. A platelet substitute, according to claim 32, wherein the chimeric protein heterodimer complex is the chimeric protein heterodimer complex stated in claim 2.

34. A platelet substitute, according to claim 33, wherein the chimeric protein heterodimer complex is the chimeric protein heterodimer complex stated in claim 6.

35. A platelet substitute, according to any one of claims 26 through 34, wherein the extracellular matrix receptor is bound to a carrier when used.

36. A platelet substitute, according to any one of claims 26 through 35, which is hemostatic.

37. A method for testing the binding between a chimeric protein heterodimer complex stated in any one of claims 2 to 9, and a ligand or cells, comprising the steps of bringing a chimeric protein heterodimer complex comprising an integrin and an immunoglobulin, and a ligand or cells into contact with each other, to prepare a mixture, and measuring the amount of the chimeric protein heterodimer complex bound to the ligand or cells or the amount of the ligand or cells bound to the chimeric protein heterodimer complex.

38. A method for searching for a substance capable of being bound to an integrin, comprising using a chimeric protein heterodimer complex stated in any one of claims 2 though 9.

39. A substance capable of being bound to an integrin, obtained by using the method stated in claim 38.

40. A method for searching for a substance which inhibits the binding between an integrin and a ligand, comprising using the method stated in claim 37.

41. A method, according to claim 40, wherein the ligand is a fibronectin fragment identified as sequence No. 3 or a collagen.

42. A protein, peptide or low molecular weight compound which inhibits the binding between an integrin and a ligand, obtained by using the method stated in claim 40 or 41.

43. A method for measuring the amount of a ligand of an integrin, comprising using a chimeric protein heterodimer complex stated in any one of claims 2 through 9.

44. A method for identifying an extracellular matrix exposed region, comprising using a chimeric protein heterodimer complex stated in any one of claims 2 through 9.

Fig. 1

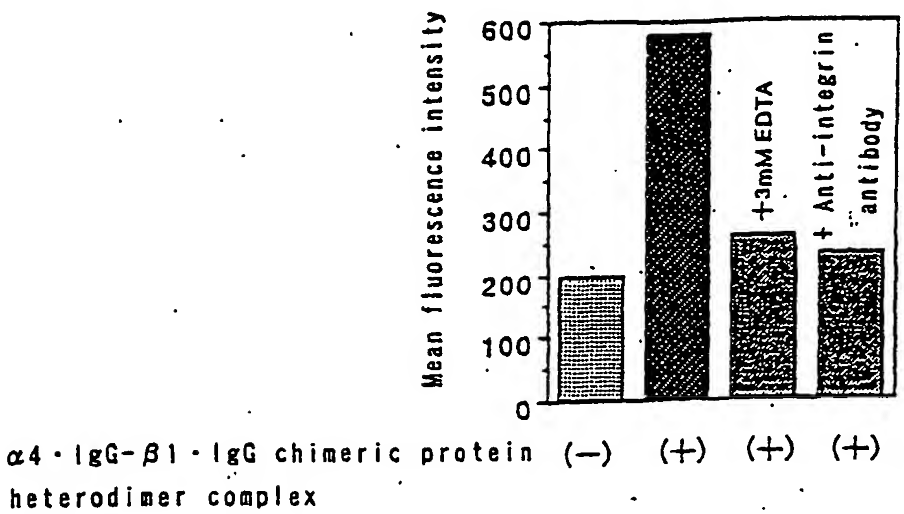

Mean fluorescence intensity
600
500
400
300
200
100
0
+3mM EDTA
+ Anti-integrin antibody
α4・IgG-β1・IgG chimeric protein heterodimer complex
(−)
(+)
(+)
(+)

Fig. 2

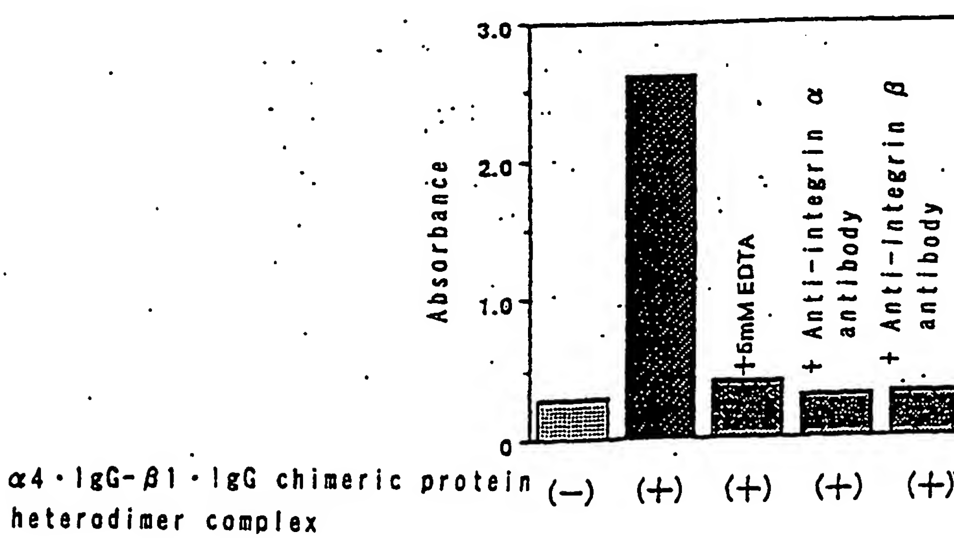

Absorbance
3.0
2.0
1.0
0
+5mM EDTA
+ Anti-integrin α antibody
+ Anti-integrin β antibody
α4・IgG-β1・IgG chimeric protein heterodimer complex
(−)
(+)
(+)
(+)
(+)

Fig. 3

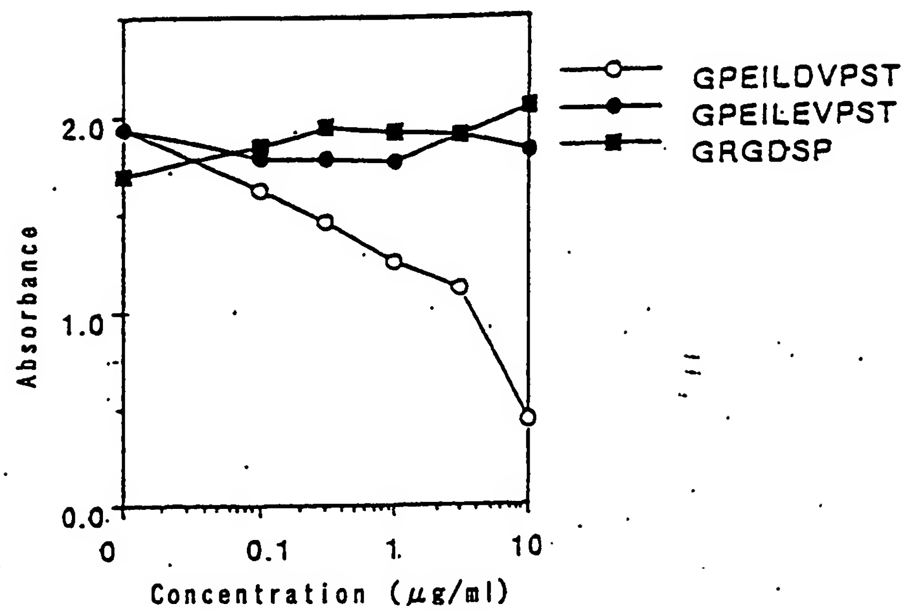

2.0
1.0
0.0
Absorbance
0
0.1
1
10
Concentration (μg/ml)
GPEILDVPST
GPEILEVPST
GRGDSP

Fig. 4

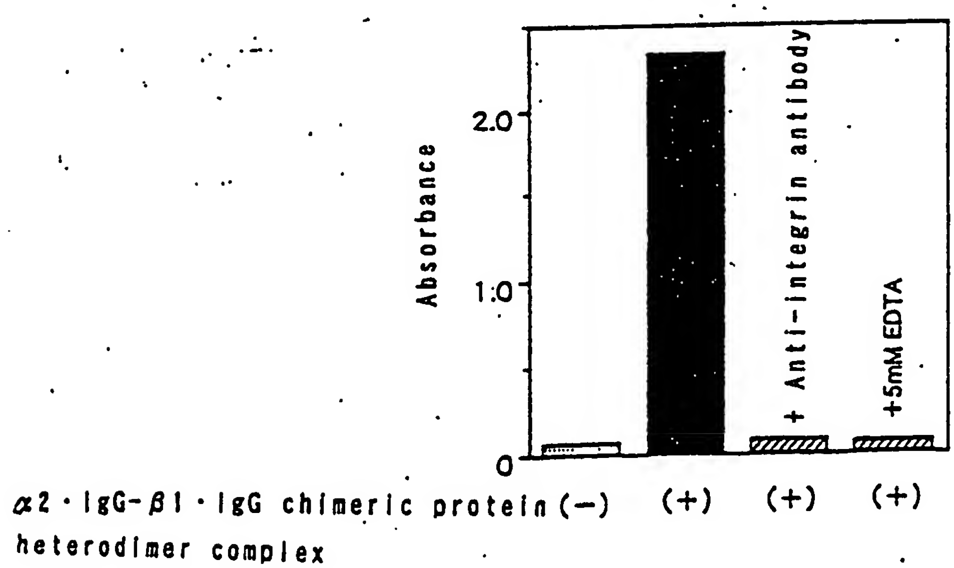

2.0
1.0
0
Absorbance
+ Anti-integrin antibody
+5mM EDTA
α2・IgG-β1・IgG chimeric protein (−) (+) (+) (+)
heterodimer complex

Fig. 5

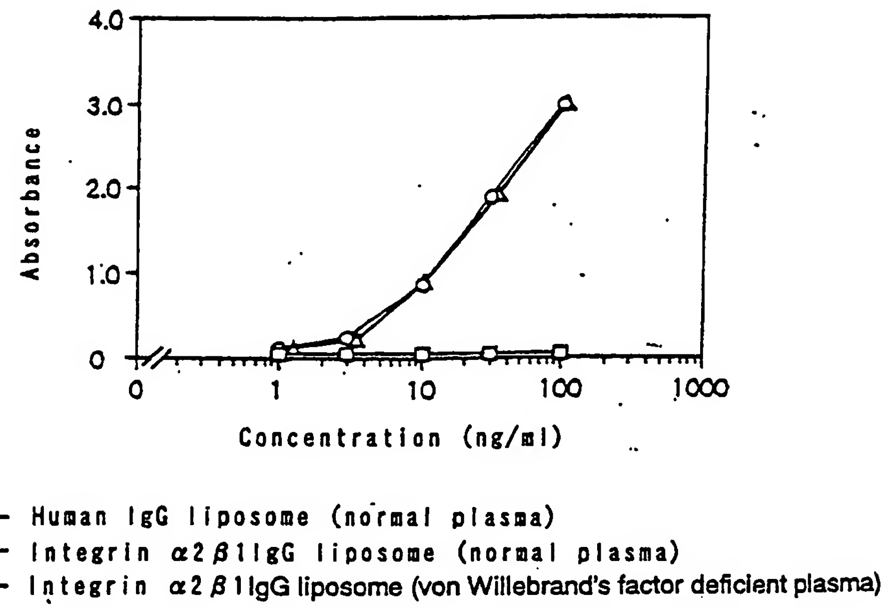
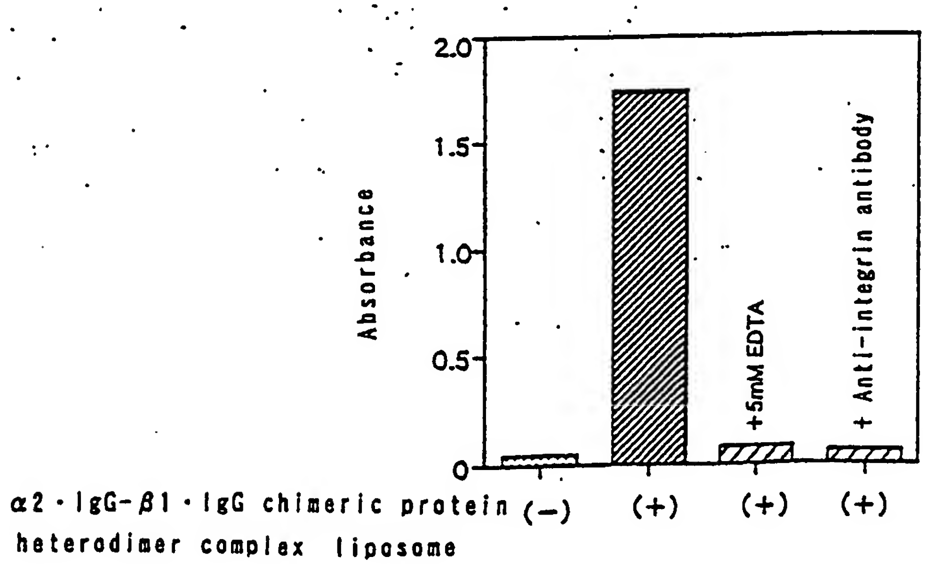

4.0
3.0
2.0
1.0
0
Absorbance
0
1
10
100
1000
Concentration (ng/ml)
Human IgG liposome (normal plasma)
Integrin α2β1IgG liposome (normal plasma)
Integrin α2β1IgG liposome (von Willebrand's factor deficient plasma)

Fig. 6


2.0
1.5
1.0
0.5
0
Absorbance
+5mM EDTA
+ Anti-integrin antibody
α2・IgG-β1・IgG chimeric protein (−) (+) (+) (+)
heterodimer complex liposome

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP98/00370

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[6] C07K14/705, C12N15/12, G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[6] C07K14/705, C12N15/12, G01N33/50

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
BIOSIS (DERWENT), WPI (DERWENT), GenBank/EMBL (geneseq)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, 92/13559, A1 (PROGENICS PHARMACEUTICALS, INC.), August 20, 1992 (20. 08. 92) & AU, 9214385, A	1-25, 26-34, 37-38, 40, 41
Y	WO, 95/02421, A1 (ALKERMES INC.), January 26, 1995 (26. 01. 95) (Family: none)	1-25, 26-34, 37-38, 40, 41
Y	JP, 6-87899, A (Educational Foundation Fujita Gakuen), March 29, 1994 (29. 03. 94) & EP, 466505, A2 & US, 5475100, A	1-25, 26-34, 37-38, 40, 41
Y	Nucleic Acids Res., Vol. 10, No. 13 (1982) J.W. Ellison et al., "The nucleotide sequence of a human immunoglobulinC $_{\gamma 1}$ gene" p.4071-4079	1-25, 26-34, 37-38, 40, 41
Y	Cell, Vol. 29, No. 2 (1982) N. Takahashi et al., "Structure of human immunoglobulin gamma genes: implications for evolution of gene family", p.671-679	1-25, 26-34, 37-38, 40, 41

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
June 8, 1998 (08. 06. 98)	June 16, 1998 (16. 06. 98)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No. PCT/JP98/00370

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EMBO J., Vol. 8, No. 5 (1989) Y. Takada et al., "The primary structure of the α^4 subunit of VLA-4: homology to other integirns and a possible cell-cell adhesion function", p.1361-1368	1-25, 26-34, 37-38, 40, 41
Y	Mol. Immunol., Vol. 32 (1995) M.C. Szabo et al., "Identification of two variants of the human integrin α_4 subunit", p.1453-1454	1-25, 26-34, 37-38, 40, 41
Y	J. Cell Biol. Vol. 105 (1987) W.S. Argraves et al., "Amino acid sequence of the human fibronectin receptor", p.1183-1190	1-25, 26-34, 37-38, 40, 41
Y	US, 5516634, A (Newman P.J.), May 14, 1996 (14. 05. 96) (Family: none)	1-25, 26-34, 37-38, 40, 41
Y	J. Cell Biol., Vol. 109 (1989) Y. Takada et al., "The primary structure of the VLA-2/collagen receptor α^2 subunit (platelet GPIa): homology to other integrins and the presence of a possible collagen-binding domain", p.397-407	1-25, 26-34, 37-38, 40, 41
Y	J. Clin. Invest., Vol. 92 (1993) S. Santoso et al., "The human platelet alloantigens Br^a and Br^b are associated with a single amino acid polymorphism on glycoprotein Ia (Integirn subunit α 2)", p.2427-2432	1-25, 26-34, 37-38, 40, 41
X/Y	JP, 7-500721, A (The Regents of the University of California), January 26, 1995 (26. 01. 95) & WO, 92/12236, A1	39/37-38, 43, 44
X/Y	JP, 5-505179, A (La Jolla Cancer Research Foundation), August 5, 1993 (05. 08. 93) & WO, 91/09874, A & EP, 507836, A & US, 5169930, A	39/37-38, 43, 44
Y	JP, 5-502228, A (Scripps Clinic & Research Foundation), April 22, 1993 (22. 04. 93) & WO, 91/07977, A & EP, 502124, A & US, 5196511, A	26-36, 42

Form PCT/ISA/210 (continuation of second sheet) (July 1992)